ON THE SIGNIFICANCE OF THE SUBMILIARY MYOCARDIAL NODULES OF ASCHOFF IN RHEUMATIC FEVER.*

By WILLIAM THALHIMER, M.D., and M. A. ROTHSCHILD, M.D.

(*From the Pathological Laboratory of the Mount Sinai Hospital, New York.*)

Plates 46 to 48.

Aschoff, in 1904, described what he considered a specific microscopic lesion in the myocardium of cases of acute rheumatic fever. Previously Romberg and his pupils had noted that interstitial changes and inflammatory foci occur in the myocardium of cases succumbing either to the acute fever or to chronic valvular disease of rheumatic origin. Aschoff called the focal lesions "submiliary nodules of rheumatic fever," and stated that they occur near the small or medium sized vessels, to the adventitia of which they often have close relation. The lesions consist of large cells with one or more large polymorphous nuclei, the cells being arranged in the form of a fan or rosette. The central portion of the nodule is composed of faintly staining, apparently necrotic masses of protoplasm. The giant cells, so called, resemble the large cells of certain sarcomatous or pseudoleukemic growths. The nodules include also large and small lymphocytes and polymorphonuclear leucocytes. The small lymphocytes are numerous at the periphery. Aschoff believes that the giant cells arise as a result of swelling from the adventitial "*Wanderzellen.*" These cells give the cell masses their characteristic appearance.

Geipel studied seven cases of rheumatic myocarditis, of which one was complicated by staphylococcus abscesses in the myocardium, and another by gonococcus endocarditis. The nodules occurred in the intermuscular connective tissue, especially about the blood vessels. He describes their origin as follows: "There is first an increase in the size of the connective tissue cells, both the nuclei and the cytoplasm taking part in the process. Later the nuclei subdivide and in some cases the cells become confluent. As a result, giant cells, 15 to 23 microns in size, develop, and these assume a peripheral arrangement about the connective tissue fibers, the latter staining faintly in the center of the nodules." Geipel's view of the origin of the nodules, namely from the interstitial connective tissue, is in contradistinction to Aschoff's view of their origin from the adventitial "*Wanderzellen.*"

Geipel also made a study of the blood vessels in other organs and failed to find any lesions resembling the Aschoff bodies. He believes that the rheumatic virus exerts a characteristic effect upon the cardiac interstitial tissue, that the

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latter undergoes a certain degree of necrosis, followed by a form of productive inflammation which exhibits itself in the formation of the Aschoff bodies. He does not admit, however, that the Aschoff bodies are specific for rheumatic myocarditis, for he claims to have observed similar nodules in the heart of a case of chronic interstitial nephritis. But as adherent pericardium coëxisted, there is the possibility, as suggested by Aschoff, of a previous rheumatic infection.

Coombs demonstrated the bodies in several series of cases, chiefly in the wall of the left ventricle. They were absent from non-rheumatic cases. Wächter reported a case in which the bodies were abundant, and Bracht and Wächter later found the nodules in three cases of acute articular rheumatism. However, in a fourth case, giving a rheumatic history, the bodies were absent. In two of the cases a *Streptococcus mitis*[1] type of organism was cultivated from the heart's blood post mortem. They failed to reproduce the lesions in animals. Saigo observed the bodies in five hearts from persons giving a previous history of rheumatism. In one instance, a woman, dying of sarcoma of the uterus, who had suffered from acute articular rheumatism twenty-seven years previously, scars with an occasional giant cell were present, a finding which he considered sufficient proof of a previous rheumatic infection. Takayasu and Löw each found the nodules in one case. Fraenkel, in order to test the specificity of the Aschoff bodies, made post-mortem studies of twenty cases in which blood cultures had been made *intra vitam*. He demonstrated the value of the Unna-Pappenheim methyl-green pyronin stain for differentiating them from other foci. Eight of his twenty cases had given a rheumatic history. In seven cases no history was available and five subjects had not suffered from rheumatism. One instance was similar to one of ours, there being a superadded vegetative endocarditis due to an infection by *Streptococcus mitis*. Of the eight cases in which a definite history of rheumatism was obtainable, five showed Aschoff bodies. In the remaining three cases the attack of rheumatism occurred three, seven, and twenty years respectively before death. However, fibrous scars occurred with a few characteristic cells in a case eighteen years after an attack of acute rheumatic fever. He calls attention to the occurrence in old cases of fibrous scars which infiltrate the myocardium. In the five cases in which there was no history of rheumatism, the nodules were absent in two, characteristic cells were found in two, and the cells and nodules in one. The fifth case was one of chorea without joint manifestations, and the characteristic nodules were present. In seven cases of verrucous endocarditis, without history, Aschoff bodies or their remains were present in six. Fraenkel believes that even in the absence of a history of rheumatism the finding of Aschoff bodies is strong presumptive evidence of a preceding rheumatic infection. In the hearts of patients dying from infection of the endocardium by the streptococcus, staphylococcus, pneumococcus, *Bacillus aerogenes*, or the diphtheria bacillus, Aschoff bodies could not be demonstrated.

Douglas found the bodies in one case of acute rheumatic fever, and Huzella found them in six cases of acute rheumatic fever, and in one case of chorea. Using the Unna-Pappenheim or other method of staining, the various authors in general confirm Aschoff's statement as to the appearance and composition of the

[1] This term is synonymous with *Streptococcus mitior seu viridans* (Schottmüller), *Streptococcus viridans*, *Streptococcus salivarius*, and *Streptococcus fecalis*.

foci and their relation to the blood vessels, but some share the opinion of Geipel, that the giant cells arise from the fixed cells of the adventitia.

MATERIAL.

The material studied consists of the following: three cases of acute rheumatic fever; three cases of chorea without rheumatic history; one case of combined verrucous and subacute bacterial endocarditis without rheumatic history; fourteen cases of subacute bacterial endocarditis in the active stage of the disease; five cases of subacute bacterial endocarditis in the bacteria-free stage; five cases of chronic valvular disease without rheumatic history; five cases of acute streptococcus endocarditis; one case of pneumococcus; three of gonococcus; and three of staphylococcus endocarditis.

CASES SHOWING ASCHOFF BODIES.

Cases 1, 2, and 3 of our series were cases of typical acute articular rheumatism. Blood cultures, made *intra vitam,* in case 1 were negative; in cases 2 and 3 a search for microörganisms in the heart was unsuccessful. Case 1 had three attacks of acute articular rheumatism and finally developed an acute pericarditis. Careful study of the heart showed that each attack had left its imprint in the myocardium. We found scars with an occasional giant cell of the Aschoff type,—a healed or healing lesion. A more recent stage was also present, showing an intermingling of Aschoff cells and fibroblasts. In addition very recent bodies were found in the myocardium, probably depending upon the last rheumatic manifestation, namely, pericarditis.

Case 2 shows the most extensive involvement of the myocardium in our series. Several Aschoff bodies are found in each microscopical field. Case 4 corresponds to a case which Fraenkel reported (case 9) in which there was no definite history of rheumatism. Blood cultures during life showed *Streptococcus mitis;* however, post-mortem examination showed both verrucous and vegetative endocarditis. The mitral valve and myocardium showed numerous bodies in the stage of fibrosis; besides this, lesions identical with those produced experimentally by Bracht and Wächter are present.[2] We would interpret this as a rheumatic process with an

[2] Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.,* 1914, xix, 429.

added bacterial involvement, as previously damaged valves are prone to secondary infections. The fact that the bodies were not present in any of the fourteen cases of subacute bacterial endocarditis reported in this paper leads us to agree with Fraenkel that even in the absence of a history of rheumatism, the finding of the bodies is strong presumptive evidence of a previous rheumatic infection.

Cases 5, 6, and 7 had a history of chorea and are of interest because the pathological lesion proves the theory which clinicians have long held, that rheumatism and chorea are closely related. None of the cases had any articular manifestations. The myocardium in case 5 showed numerous bodies with a considerable fibrosis and in addition a diffuse infiltration with Aschoff cells. The sudden cardiac death in this case might possibly be explained by the diffuse invasion of the myocardium. Cases 6 and 7 showed only a few small bodies. In none of these cases have we been able to demonstrate bacteria in the lesions with either the Gram-Weigert, cresyl-violet, or Unna-Pappenheim stains.

Case 1.[3]—C. R., age 17 years, had been a patient in Mount Sinai Hospital three times previously with acute articular rheumatism and a valvular defect. The patient returned to the hospital with dyspnea, palpitation, and fever.

Autopsy. Anatomical Diagnosis.—Verrucous endocarditis of mitral and aortic valves; aortic stenosis and insufficiency; cardiac hypertrophy and dilatation; old and recent fibrous pericarditis; fibrous pleurisy (left). Heart weighed 810 gm. The tricuspid orifice is slightly thickened and along its line of closure there are scattered numerous small verrucæ varying from 1 to 3 mm. in size. The mitral valve is dilated and presents numerous verrucæ; mitral ring calcified; the cusps of the aortic valve are markedly thickened and along the free edge of the cusps are numerous verrucous projections.

Microscopical Examination. Heart.—Section stained with methyl-green pyronin. Great numbers of Aschoff bodies and diffuse fibrosis of the myocardium. The bodies are found in the wall of the left ventricle, left papillary muscles, interventricular septum, about the calcified mitral ring, beneath the endocardium, and in the mitral valve, but not in the auricles.

Three main types of this lesion occur: (1) Collections of a few typical cells not more than eight to ten in number, closely packed against one another, immediately around small blood vessels. (2)

[3] Case 1 was studied through the kindness of Dr. Alfred Meyer, of Mount Sinai Hospital.

Large collections of cells, numbering thirty or forty, arranged in a loose network of fibroblasts and young connnective tissue cells. These occur mainly about the arteries, usually those of large or medium size, and beneath the endocardium. Some of the collections resemble a rosette in form, but most of them are fusiform accumulations in the adventitia of the blood vessels, and many may be found on both sides of the blood vessel as though completely encircling it. (3) A small number of perivascular foci made up of dense fibrous tissue with a few large typical giant cells at their center.

The greatest number of the bodies are found about the mitral ring, where in the midst of a dense fibrous tissue, which is infiltrated with calcium, are found typical discrete collections and large masses of scattered cells. Many plasma cells and large mononuclear leucocytes and a few polymorphonuclear leucocytes are found here and elsewhere about the bodies, together with numerous fibroblasts which take a bright red stain somewhat different from that of the giant cells, some of which are drawn out, flattened, or fusiform in shape. The mitral valve itself shows a number of the bodies mainly of the second type described, and in addition a dense fibrosis, fibroblasts, and mononuclear and plasma cells. The blood vessels everywhere, especially the small and medium sized ones, show a moderate intimal thickening while in some places there seems to be a narrowing of the lumen opposite the Aschoff bodies.

Case 2.[4]—R. H., age 17 years; negro, male. Admitted to the City Hospital, February 17, 1913; died, February 23, 1913. Patient complained of pain and swelling of ankles and knees. *Past history.*—Rheumatism five years ago with swelling of joints. *Present illness.*—Began about a week before admission with pain and swelling of ankles, later of both knees.

Autopsy.—Fibrous hypoplastic aorta; lymphatic hyperplasia of spleen and mesenteric lymph nodes; persistent thymus; fibrous pericarditis. *Heart.*—Weight 300 gm. The whole heart is encased in a dry, fibrinous exudate; valves normal. Left ventricle measures 9 mm. in thickness. On section the myocardium is normal in color, and shows no areas of sclerosis. Coronary arteries normal; aorta narrow, and free from arteriosclerosis.

Microscopical Examination. Heart.—Section stained with methyl-green pyronin. The heart contains a great number of Aschoff

[4] Case 2 was studied through the kindness of Dr. John Larkin, of the City Hospital, New York.

bodies, there being several in almost every microscopic field. Practically all the bodies are large, containing twenty to forty cells; they are arranged beneath the endocardium, in the adventitia of the blood vessels, and between the muscle fibers apparently independently of blood vessels. About half the cells are of the large multinuclear type, of which some contain five or six nuclei. The arrangement is loose, not densely packed together as in case 1. Between some of the cells is a pink staining matrix which contains many fine fibrils (fibrin?). In other bodies the cells are separated by a fibroblastic kind of tissue.

Case 3.[5]—J. S., age 23 years; white, female; married. Admitted to the City Hospital, January 23, 1913; died, March 5, 1913. Patient complained of headache, pain in joints and chest. *Past history.*—Scarlet fever when a child; typhoid one year ago. *Present illness.*—Began about three months before admission. The patient died forty-one days after admission to the hospital. Cardiac murmur remained present and unchanged with the exception of pericardial friction rub which appeared for a few days and then disappeared. Patient developed dullness on both sides of the chest posteriorly about two weeks before death. The chest was tapped and large quantities of clear fluid were removed on two occasions. This fluid was cultured twice, and cultures and spreads were negative. Three blood cultures were taken and all were negative. Wassermann reaction was negative.

Autopsy.—Only a partial autopsy was obtained. *Heart.*—Slightly hypertrophied and showed a slight chronic thickening of all its valves, and a few typical small pin-head verrucæ along their edges.

Microscopical Examination.—Section stained with methyl-green pyronin. The heart contains only a few typical Aschoff bodies in the adventitia about several medium sized or larger arteries. The bodies contain from six to ten typical cells, some of which are large and multinuclear; most of them are slightly larger than large mononuclear leucocytes and contain a single nucleus. The cells which are chiefly elongated have no definite arrangement and are separated from one another by the connective tissue of the adventitia; they lie with their long axis parallel to the direction of the blood vessel.

Case 4.[6]—M. W., age 17 years. Admitted to Mount Sinai Hospital, June 16, 1913; died, June 23, 1913. Onset of illness six weeks before admission, with headache, epistaxis, vomiting, generalized pain. Satisfactory history unobtainable. *Streptococcus mitis* (*viridans*) was recovered by blood culture.

[5] Case 3 was studied through the kindness of Dr. John Larkin, of the City Hospital, New York.

[6] Case 4 was studied through the kindness of Dr. J. Rudisch, of Mount Sinai Hospital.

Autopsy. Anatomical Diagnosis.—Subacute aortic and mitral bacterial endocarditis; verrucous mitral endocarditis and mitral stenosis; infarcts in the spleen and kidney; embolic glomerular lesions. *Heart.*—Normal in size; wall of left auricle increased in thickness; mitral valve is narrowed; the flaps are thickened and fibrous and continue downward onto the chordæ tendinæ for a slight distance, forming a funnel-shaped orifice, the free edge ending in a slight projecting shelf beyond the chordæ. Along the free edge of this shelf-like projection, very close to one another, are many smooth, pin-head verrucæ which are translucent in appearance. They are firm and crush with difficulty. On the ventricular surface of the aortic flap of the mitral valve are two large pedunculated cauliflower-like vegetations about half a centimeter in width and projecting for a distance of one centimeter. They are mixed red and gray in color and are friable. The cusps of the aortic valve show a slight irregular fibrous thickening. The ventricular surface of the cusps contains numerous small warty vegetations similar to those around the base of the large mitral vegetation. One of the cusps of the aortic valve, which is covered by vegetations, comes in contact with the large mitral vegetation when in the closed position.

Smears from the large mitral vegetation and those on the aortic valve show a moderate number of minute gram-positive cocci, which in culture proved to be *Streptococcus mitis* (*viridans*). Smears and cultures, from crushed verrucæ on the mitral valve, were negative.

Microscopical Examination.—Sections stained with hematoxylin and eosin. Section of auricular-ventricular junction, mitral valve, left ventricle, and papillary muscle. The section shows diffuse round cell infiltration, slight edema, and patchy increase in interstitial tissue. Within the fibrous patches and between the muscle fibers, focalized collections of round cells giving the same picture as the lesions produced experimentally by Bracht and Wächter occur, including atrophy and degeneration of muscle fibers. In the adventitia of a number of the large and medium sized vessels and also in the myocardium between the muscle fibers are seen a moderate number of irregular nodular collections of cells, three to four times the size of large mononuclears, of which some are elongated and others multinuclear. The nuclei are vesicular, the protoplasm is finely granular, while between some a material which is either coagulated serum or fibrin occurs. The mitral valve shows fibrosis, slight round cell infiltration and small, irregular projections corresponding to the verrucæ, which are composed of more recent fibrous tissue. The blood vessels are thickened and show round cell infiltration.

In sections stained with methyl-green pyronin the nodular collections of cells just described take a brilliant dark red stain and are

seen to be typical Aschoff bodies. Others occur beneath the endocardium, and a few in the papillary muscle. The verrucæ at the edge of the mitral valve are made up of young connective tissue network. Throughout these small rounded projections are seen brilliant, dark red staining cells, the protoplasm of which is either homogeneous or finely granular. The cells are either fusiform, and three to four times the size of large mononuclear cells, or present long, interlacing, streamer-like processes. The nuclei are large and poor in chromatin. The first cells are suggestive of the cells of the Aschoff bodies, the latter having the appearance of fibroblasts.

Case 5.[7]—F. B., age 10 years. History of chorea at the age of 7; three attacks before the present admission; child died with symptoms of meningitis; clear cerebrospinal fluid.

Autopsy.—The pericardium is densely adherent to the sternum and when opened a large quantity of seropurulent fluid escaped. Parietal and visceral layers are rough, granular, and congested. *Heart.*—Both ventricles are hypertrophied; near the free margins of the tricuspid, mitral, and aortic valves are numerous small vegetations.

Microscopical Examination.—Section stained with methyl-green pyronin. The heart contains a few atypical Aschoff bodies. Many of the medium sized arteries show irregularly distributed thickening of the intima. In these arteries, diffusely scattered through the intima and extending into the adventitia are found cells resembling young fibroblasts and proliferating endothelial cells; but in the adventitia many of the cells have the typical appearance of Aschoff cells of which a number contain two or three nuclei.

Case 6.[8]—Female, age 12 years. Two weeks before the present illness the patient had tonsillitis and later complained of pain in arms and legs. One week later symptoms of chorea developed; movements became extremely violent and child became delirious, passing into coma and dying forty-eight hours later. Examination showed a few fibrous nodules along the tendons of the palms of the hands.

Autopsy. Anatomical Diagnosis.—Acute vegetative mitral endocarditis; bronchopneumonia of right lung; congestion of the pia mater. *Heart.*—Weight

[7] Case 5 was studied through the kindness of Prof. E. L. Opie, of George Washington University, and Prof. John Howland, of Johns Hopkins Medical School.

[8] Case 6 was studied through the kindness of Prof. E. L. Opie, of George Washington University, and Prof. John Howland, of Johns Hopkins Medical School.

172 gm.; endocardium normal, except at the line of closure of the mitral cusps on which is a continuous line of firmly attached vegetations.

Microscopical Examination.—Section stained with methyl-green pyronin. The heart contains a moderate number of typical Aschoff bodies present only in the adventitia of the arteries. They are made up of collections of six to forty cells which in a few instances are densely packed. In most cases the cells occur in the midst of the connective tissue, and are slightly separated from one another. The cells, of which one third are mononuclear, appear to be young, and many of them resemble fibroblasts.

Case 7.[9]—E. S., age 16 years. Admitted to Mount Sinai Hospital, Sept. 28, 1907; died, Oct. 27, 1907. Diagnosis, acute endocarditis. Gave a previous history of measles; chorea four years ago; no articular symptoms or sore throat; no history of rheumatism. For two months before admission had dyspnea and palpitation on exertion; cough, worse at night; headache.

Autopsy. Heart.—Enlarged; small areas of thickening of pericardium; walls of ventricles and auricles markedly hypertrophied. All cavities considerably dilated. Hypertrophy of papillary muscles of left ventricle. Mitral valve admits two fingers. All flaps markedly thickened. Free edges of flaps show irregular small verrucous vegetations. On the wall of the left ventricle, about two centimeters below the aortic orifice, there is a line of minute vegetations. All cusps are markedly thickened and retracted; about the free edge there are verrucous vegetations, some of which are five millimeters in diameter. The tricuspid valve admits two fingers; many vegetations occur along the thickened free edges of the cusps. The heart shows myocarditis, particularly in the left ventricle.

Microscopical Examination.—Section stained with methyl-green pyronin. The heart shows throughout numerous Aschoff bodies, most of which are large and contain as many as a hundred cells, and large, irregular areas of fibrosis in which a moderate diffuse round cell infiltration and a few polymorphonuclear leucocytes exist. The Aschoff bodies occur almost entirely in the adventitia of the medium sized and small arteries and in the midst of the areas of fibrosis. The bodies are loose collections of typical cells largely separated from one another by connective tissue fibers and containing in the center between the cells pink staining granular material. Some of the cells contain six nuclei.

[9] Case 7 was studied through the kindness of Dr. J. Rudisch, of Mount Sinai Hospital.

CASES IN WHICH ASCHOFF BODIES WERE NOT PRESENT.

We have examined the hearts of fourteen cases of subacute bacterial endocarditis,[10] of which disease descriptions have been given by Osler, Schottmüller, Horder, Libman, and others. The specimens were obtained from cases which Dr. Libman has reported. Blood cultures during life were positive in all, the invading organism being *Streptococcus mitis.* Ten of the cases gave no history of rheumatism. In none of these could Aschoff bodies be demonstrated. In only one of the four cases in which a history of rheumatism existed did we find any evidence of Aschoff cells. These cells characteristic of the bodies were few and occurred in the fibrous scars about the small vessels. This patient had suffered from an attack of acute articular rheumatism eleven years and again one year before death. Two other cases had "rheumatism" five and seven years, respectively, before death. The fourth patient had "rheumatism" with valvular disease fourteen years before admission. At the time of admission he had joint pains and was evidently suffering from a superadded bacterial infection involving his previously damaged valves. It is difficult to distinguish the arthritic manifestations in bacterial endocarditis from those of acute rheumatic fever. We have also examined the hearts of five cases of subacute bacterial endocarditis in the bacteria-free stage, as first described by Dr. Libman. Only one gave a history of "rheumatism," the attack antedating death by five years. None showed Aschoff bodies. In all these hearts lesions occurred similar to those produced experimentally by Bracht and Wächter with *Streptococcus mitis.* We have also examined with negative results the hearts of five patients that had had chronic valvular disease without rheumatism. In five cases of acute streptococcus endocarditis, one case of suppurative pericarditis accompanied by pneumococcus endocarditis, two cases of gonococcus endocarditis, and three cases of staphylococcus endocarditis, we failed to find the presence of Aschoff bodies in the heart.

CONCLUSIONS.

1. In rheumatic myocarditis, foci, termed submiliary nodules of Aschoff, are present which are characteristic of the rheumatic infection.

[10] A large series of cases is being studied and will be reported later.

2. They are most frequently found in the walls of the left ventricle, the auricles usually escaping.

3. The nodules were found in three cases of chorea without joint manifestations, proving the close relation of this condition to rheumatism.

4. They were absent in fourteen cases of subacute bacterial endocarditis due to *Streptococcus mitis.*

5. They were not found in infections of the endocardium with the gonococcus, staphylococcus, streptococcus, or pneumococcus.

6. Even in the absence of a rheumatic history we believe, in accordance with Fraenkel, that the presence of Aschoff bodies signifies a previous rheumatic infection.

7. Aschoff bodies are not always found in rheumatic carditis, where the infection antedates death by a long period, but the healed remains, represented by sclerotic patches (*"Schwielen"*), are present.

8. We suggest that the cases of arthritis characterized by the presence of the submiliary nodules of Aschoff in the myocardium be placed in one group and called for the time being "rheumatism"; and the cases with articular manifestations, yielding positive bacteriological findings and no Aschoff bodies, should be classified according to the infecting microörganisms concerned, and not as rheumatism.

BIBLIOGRAPHY.

Aschoff, L., *Verhandl. d. deutsch. path. Gesellsch.,* 1904, viii, 46.

Aschoff, L., *Brit. Med. Jour.,* 1906, ii, 1103.

Bracht, E., and Wächter, *Deutsch. Arch. f. klin. Med.,* 1909, xcvi, 493.

Coombs, C., *Brit. Med. Jour.,* 1907, ii, 1513; *Quart. Jour. Med.,* 1908–09, ii, 26; *Lancet,* 1909, i, 1377; *Jour. Path. and Bacteriol.,* 1910–11, xv, 489; *St. Mary's Hosp. Gaz.,* 1913, xix, 22.

Douglas, M., *Jour. Path. and Bacteriol.,* 1913, xviii, 119.

Fraenkel, E., *Beitr. z. path. Anat. u. z. allg. Path.,* 1912, lii, 597.

Geipel, P., *Deutsch. Arch. f. klin. Med.,* 1905, lxxxv, 75; *München. med. Wchnschr.,* 1909, lvi, 2469.

Horder, T. J., *Quart. Jour. Med.,* 1908–09, ii, 289; *Report of the Local Government Board, Supplementary Report of the Medical Officer,* London, 1906–07, xxxvi, 279.

Huzella, T., *Virchows Arch. f. path. Anat.,* 1913, ccxiii, 389.

Libman, E., and Celler, H., *Am. Jour. Med. Sc.,* 1910, cxl, 516; *Tr. Assn. Am. Phys.,* 1910, xxv, 5.

Löw, J., *Beitr. z. path. Anat. u. z. allg. Path.,* 1910, xlix, 1.

Osler, W., *Brit. Med. Jour.*, 1885, i, 467, 522, 577, 607; *Practitioner*, 1893, l, 181; *Quart. Jour. Med.*, 1908–09, ii, 219.
Romberg, E., *Deutsch. Arch. f. klin. Med.*, 1891, xlviii, 369; 1894, liii, 141.
Saigo, Y., *Beitr. z. path. Anat. u. z. allg. Path.*, 1908, xliv, 296.
Schottmüller, H., *München. med. Wchnschr.*, 1910, lvii, 617; 1903, l, 849, 909.
Takayasu, R., *Deutsch. Arch. f. klin. Med.*, 1909, xcv, 270.
Wächter, *München. med. Wchnschr.*, 1908, lv, 1101.

EXPLANATION OF PLATES.

Plate 46.

Fig. 1. Aschoff bodies, situated in the adventitia of a medium sized artery, showing typical morphology. Stained with hematoxylin and eosin. × 80.

Fig. 2. Aschoff body, situated in the adventitia of a small artery. Stained with methyl-green pyronin. × 160.

Plate 47.

Fig. 3. Left auricle with subendocardial Aschoff bodies and thickened endocardium. Stained with methyl-green pyronin. × 40.

Fig. 4. Aschoff bodies in interstitium of left ventricle. Stained with methyl-green pyronin. × 72.

Fig. 5. Aschoff body, showing multinuclear cells. Stained with methyl-green pyronin. × 224.

Plate 48.

Fig. 6. Aschoff body in interstitium of left ventricle, from case of chorea. Stained with methyl-green pyronin. × 104.

Fig. 7. Interstitial lesion of fibrous type (Bracht and Wächter) in heart showing both subacute bacterial endocarditis and verrucous endocarditis. Stained with methyl-green pyronin. × 16.

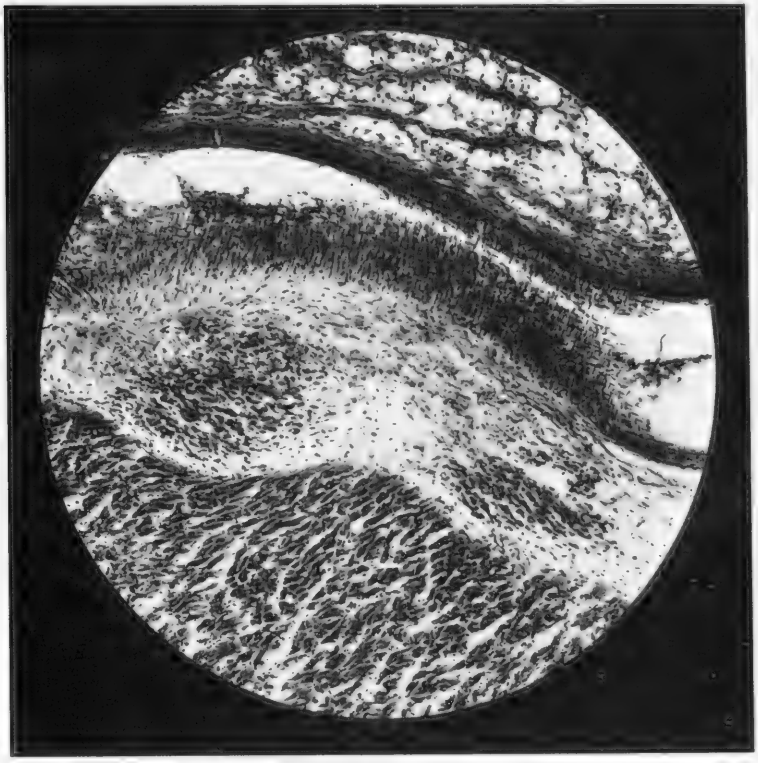


FIG. 1.

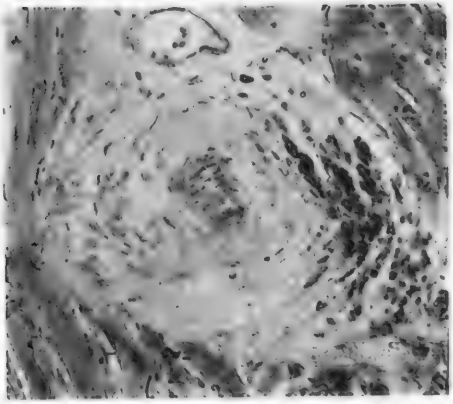


FIG. 2.

(Thalhimer and Rothschild: Nodules of Aschoff.

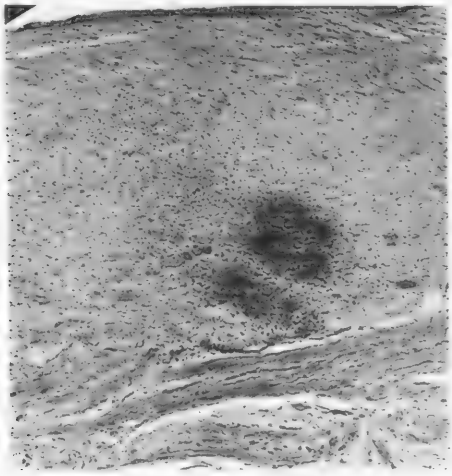


FIG. 3.

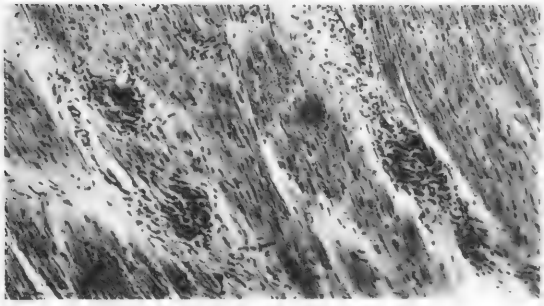


FIG. 4.

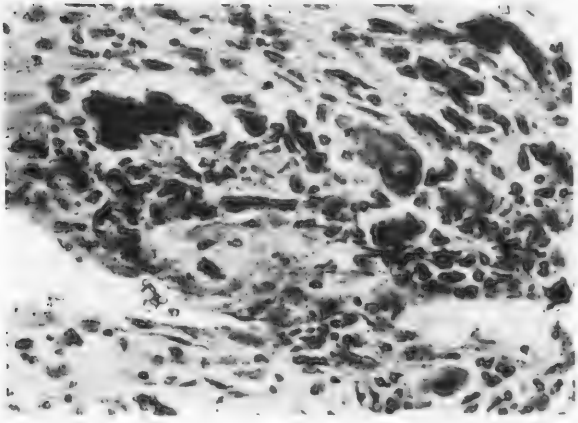


FIG. 5.

(Thalhimer and Rothschild: Nodules of Aschoff.)

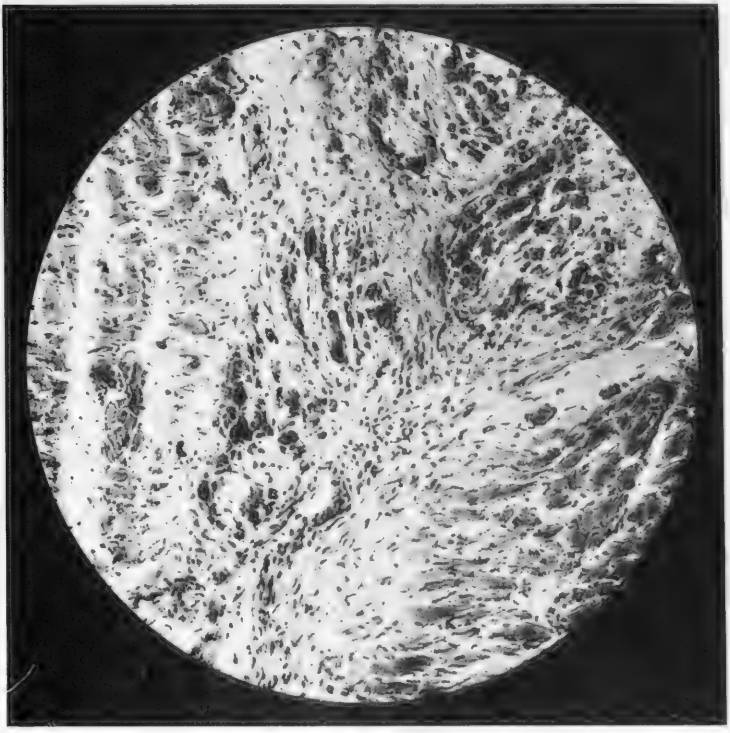


FIG. 6.

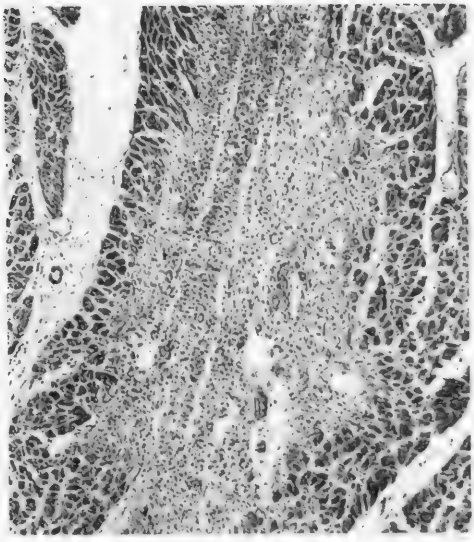


FIG. 7.

(Thalhimer and Rothschild: Nodules of Aschoff.)

EXPERIMENTAL FOCALIZED MYOCARDIAL LESIONS PRODUCED WITH STREPTOCOCCUS MITIS.*

By WILLIAM THALHIMER, M.D., and M. A. ROTHSCHILD, M.D.

(*From the Pathological Laboratory of the Mount Sinai Hospital, New York.*)

Plates 49 and 50.

The involvement of the heart in the pathological processes of acute rheumatic fever has long been known. It takes the form of endocarditis, pericarditis, and myocarditis, either alone or in combination. Of these the submiliary myocardial nodules of Aschoff alone seem to be specific. Because of the resemblance of acute rheumatism to diseases of an infectious nature the search for the etiological agent of rheumatism and its associated conditions has centered about a bacterial cause.

The literature on this phase of the subject has been reviewed by Poynton and Paine, Pribram, Sanderson, Menzer, de Vecchi, and others. The different observers may be classified into two groups according to the scheme of de Vecchi: first, those who using a perfect technique failed to cultivate bacteria from the blood or joints of these cases and who do not believe in a bacteriological etiology; and second, those who have succeeded in recovering bacteria. Different bacteria have been found by the observers belonging to the second group. Some, mainly the older investigators, believe that rheumatism is caused by common, pyogenic bacteria. Leyden, Poynton and Paine, Rosenow, and others have recovered a diplostreptococcus from cases which they have classified as rheumatism. Still another group, mostly of the French school, have cultivated a Gram-negative, anaerobic bacillus. Finally, other authors have considered this disease a mixed infection of cocci and bacilli, perhaps not even specific.

Our interest in this subject was aroused by the statements of Meyer, Shaw, and Major concerning the identity of the cultural characteristics of *Streptococcus rheumaticus* (of Leyden, Triboulet, Meyer, Wassermann, Poynton and Paine, and others) and *Streptococcus viridans* (Schottmüller), better named, in our opinion, *Streptococcus mitis*. The last named organism has been isolated in this laboratory from the blood of cases of subacute bacterial endocarditis which have been studied extensively by Dr. Libman.[1]

* This work was done under the tenure of a George Blumenthal, Jr., Fellowship. Received for publication, February 3, 1914.

[1] The cultural characteristics of these organisms will not be discussed here, since studies of this entire group of streptococci will soon be published by Drs. Libman and Aschner.

Poynton and Paine and Rosenow are the leading advocates of the theory that *Streptococcus rheumaticus* is the logical cause of rheumatism. The former base their arguments on two main facts: first, the recovery of this variety of diplostreptococcus from the joints, urine, tonsils, subcutaneous nodules, etc., from the cases classified by them as rheumatism; and second, the production in rabbits of endocarditis, pericarditis, myocarditis, arthritis, chorea (?), etc., which they consider the counterpart of the manifestations of rheumatism. Numerous workers have shown, however, that other types of bacteria can produce carditis. The above named authors have not definitely shown the identity of the experimental and the human lesions. Horder studied a comparatively large series of cases of acute rheumatic fever and malignant endocarditis, and whereas he was able to recover by blood culture a diplostreptococcus, which appeared to him to be identical with *Streptococcus rheumaticus,* from 90 per cent. of the cases of malignant endocarditis (*Bacillus influenza,* etc., from the remainder), he was unable to recover any bacteria from the blood or joint exudate of cases of acute articular rheumatism. He came to the conclusion that the contradictory results of the various observers were not due to insufficient bacteriological technique, but rather to a discrepancy in classification, the cases from which the *Streptococcus rheumaticus* was isolated being instances of malignant endocarditis and not rheumatism, or, as they are called by Poynton and Paine, "malignant rheumatism."

Rosenow has produced similar lesions in rabbits injected intravenously with large doses of an organism resembling *Streptococcus viridans* (Schottmüller), isolated by blood culture from cases of chronic infective endocarditis, and by similar organisms recovered from the tonsils. Rosenow and Coombs have described myocardial lesions in these rabbits which they consider similar to Aschoff bodies, but which in our opinion do not resemble these bodies. Rosenow has also recovered three types of a diplostreptococcus from the blood and joint exudates of a series of cases of rheumatism. These organisms, he claims, when recently isolated have on intravenous injection in rabbits an affinity for the joints though they also cause cardiac lesions. Rosenow considers this work a confirmation of that of Poynton and Paine, although in his cultures the streptococci grew in the original culture only in small numbers and under partial anaerobic conditions, whereas the latter workers recovered their organisms in large numbers and under ordinary aerobic conditions.

Bracht and Wächter, working with a similar type of *Streptococcus mitis,* caused by intravenous injection in rabbits definite myocardial lesions identical with those later described in this paper. They considered these lesions entirely different from Aschoff bodies. De Vecchi, who looks with scepticism on the importance of all bacterial findings in their primary relation to rheumatism, made a number of experiments by injecting blood from rheumatic cases into several species of animals. He described peculiar perivascular myocardial lesions containing a peculiar type of large mononuclear leucocyte, but having no particular resemblance to Aschoff bodies. His work, however, is not convincing.

Coombs, experimenting with *Streptococcus rheumaticus,* claims to have produced Aschoff bodies in rabbits, but the lesions he describes resemble more those described by Bracht and Wächter than they do those of Aschoff. Jackson produced focalized myocardial lesions in rabbits by intravenous injection of strepto-

cocci isolated from the sore throat epidemic in Chicago. These animals developed arthritis and some of them endocarditis. The myocardial lesions are those of a combined exudative and productive inflammation and have some points of resemblance to those produced by Bracht and Wächter. They do not conform to Aschoff bodies.

EXPERIMENTAL.

The present work was undertaken to ascertain the cardiac lesions produced by intravenous injections of *Streptococcus mitis,* and not to investigate this organism from the point of view of the etiological agent of rheumatism. For comparison inoculations were made with *Streptococcus rheumaticus, Streptococcus hemolyticus,* and a streptococcus isolated from cases of epidemic sore throat. Since Poynton and Paine, Rosenow, and others recovered a diplostreptococcus from cases of acute rheumatic fever, and Jackson and Coombs claim to have produced lesions similar to the submiliary myocardial nodules of Aschoff by inoculating streptococci, attention has been directed to the relation, if any, between the myocardial lesions arising in our experiments and the Aschoff bodies.

Organisms Used.—Nine different strains of *Streptococcus mitis* were used (Nos. 4,110, 4,147, 4,170, 4,206, 4,275, 4,342, 4,357, 2,524, and organism C). Organism C was isolated from the tonsils of a case of phlegmon of the neck; the other organisms were obtained by blood culture from cases of subacute bacterial endocarditis studied by Dr. Libman. All except No. 2,524 were recently isolated strains, often only one or two cultures removed from the original blood cultures, and resembled the *Streptococcus viridans* of Schottmüller. The growth on agar adhered to the surface of the medium, while in bouillon the organisms grew in clumps and adhered to the sides of the tube. Organism 2,524 was an old strain and had been in the laboratory for three years, being transplanted on glucose serum agar every four days.

The miscellaneous cultures consisted of five strains of *Streptococcus rheumaticus,* and three strains of epidemic streptococci.[2]

[2] We are indebted to Dr. Winslow, of the American Museum of Natural History, New York, for No. 34 (Poynton and Paine), No. 259 (Beattie), and No. 399 (Lintz), and to Dr. Rosenow, of the Memorial Institute, Chicago, for Nos. 735 and 736. Dr. Rosenow kindly furnished us with three strains of streptococci isolated from the Chicago sore throat epidemic, Nos. 211, 214, and 233, which had been studied by Jackson.

The organisms were grown on serum agar, serum glucose agar, human blood agar, and plain bouillon. Thick saline suspensions were made of the twenty-four hour agar cultures and the growth in bouillon was concentrated by centrifugalization. In some instances rabbits were inoculated with blood containing innumerable bacteria taken from another animal with vegetative endocarditis.

The dose varied from the twenty-four hour growth on one slant agar tube to that on two Blake bottles, or the centrifugalized sediment of a twenty-four hour growth in from 10 to 100 cubic centimeters of bouillon. Rabbits received from one to sixteen injections at intervals of from three days to several weeks.

The rabbits were of different ages, young, half grown, or old, and came from several stocks, in the attempt to rule out any special susceptibility or resistance in any one strain. One monkey was also employed.

STREPTOCOCCUS MITIS.

Forty-two rabbits were inoculated with *Streptococcus mitis*. All showed the microscopic lesions described below. Four were killed 1, 3, 4, and 7 days after injection, and a few died within the first week, but most lived for a long period, and one for as long as six months. Many of the animals developed arthritis and became emaciated, and those that lived longest developed a scaly condition of the skin of the face and paws. Only three rabbits (7 per cent.) developed vegetative endocarditis, a much smaller proportion than that reported by Rosenow. Different media, and in some instances organisms only two generations removed from the original blood culture, were used. Rabbit blood agar was not used;[3] and whether this difference is responsible for the smaller percentage of endocardial localizations can only be conjectured.[4] Nine rabbits (21 per cent.) developed hemorrhages in the valves.[5] The only rabbits that showed a bacteriemia were those with vegetative endocarditis.

[3] At present this work is being repeated by one of us (Thalhimer) in conjunction with Celler, using organisms grown on rabbit blood agar.

[4] The same result was observed by Drs. Libman and Celler, in 1903 (unpublished), who inoculated a large number of rabbits, of which a small percentage developed endocarditis.

[5] Hemorrhages in the cardiac valves are considered by Rosenow to be the earliest stage of an endocarditis caused by *Streptococcus mitis*.

Even those dying a few days after injection showed sterile blood at autopsy. In a few animals blood cultures were taken from the ear vein of the opposite ear to that injected at intervals of 1, 5, 10, 30, and 60 minutes after injection. The number of colonies decreased rapidly with the lengthening of the interval and none were found after ten minutes had elapsed.

The urine of a considerable number of the rabbits was examined. In none did indications of spontaneous albuminuria occur during the period of observation before injection.

Many of the animals developed after injections a transitory albuminuria with casts, and several showed this condition constantly after many injections. Two animals became anuric for forty-eight hours, one just before death. During the anuria the animals were drowsy, but when a sudden noise was made, they would suddenly jump in a spasmodic fashion as though slightly uremic.[6] Renal lesions, which are being investigated further, were found in this series of animals.

A monkey received seven injections of the contents of a twenty-four-hour growth on four Blake bottles of serum glucose agar; it showed myocardial lesions identical with those in the rabbit.

Myocardial Lesions.—In most cases the hearts showed no macroscopical lesions, except those mentioned; but in a few instances grayish streaks existed in the myocardium. Viewed microscopically a progressive type of lesion was present, depending on the number of injections and the length of time the animal lived.

The earlier stage of the lesion is represented by small areas including only a few muscle fibers showing slight degenerative changes, and between and about them a few infiltrating lymphocytes, large mononuclear leucocytes, occasional plasma cells, and rarely a few polymorphonuclear leucocytes. In animals receiving many injections the areas are larger, and in them the degenerating fibers show at times marked fragmentation, while the surrounding fibers are normal. The degenerating muscle fibers take a slightly more intense eosin stain than normally and the fibrils disappear early, leaving a homogeneous, finely granular protoplasm with small

[6] This condition has been reported by several observers, and may have been interpreted as chorea in rabbits.

and large vacuoles. Some of the fibers are swollen, but most are atrophic. The nuclei are irregular and either pale and vesicular, or small and pyknotic, and are not proliferating.

In addition to these more common lesions some of the hearts which show grayish myocardial streaks show microscropically areas of complete necrosis of the muscle fibers with calcareous infiltration and a slight round cell inflammatory reaction about them. With the atrophy of the muscle fibers, the interstitial tissue stands out more prominently and very early begins to proliferate, fibroblasts appearing. These areas are irregular in size and shape and are usually elongated, with the long diameter parallel to the muscle fibers. From this stage on the lesions are proliferative rather than infiltrative leucocytic or degenerative. In none of the areas are many degenerating muscle fibers seen, but fibroblasts soon become prominent and develop rapidly to a fibrous stage. Coincidently the round cell infiltration decreases until the result is an area of fibrous tissue surrounded by healthy muscle fibers and containing a few leucocytes.

Not all the fibrous areas are circumscribed; many are diffuse, their fibers running parallel to those of the myocardium, apparently having replaced the latter. Some of the focalized lesions have a close relation to the small and medium sized blood vessels, but many lie scattered through the myocardium, the greatest numbers occurring near the base of the ventricles and in the papillary muscles. The small blood vessels in some cases show proliferation of the intima or the connective tissue immediately about them. Occasionally hyaline thrombi are found, but they do not occur regularly and appear to have no relation to the lesions. The lesions themselves show no predilection for a subendocardial situation nor for the bases of the cardiac valves; no proliferation of the endothelium of the endocardium or valves occurs. No multinuclear cells such as are described by Jackson, or peculiar cells with large vesicular nuclei and scanty protoplasm as described by de Vecchi, are present. With the methyl-green pyronin stain, on alcohol-fixed material, the fibroblasts, and in some hearts the endothelium of the blood vessels, stained a bright red, as do some of the degenerating muscle fibers. No structures at all resembling the submiliary myocardial bodies of

Aschoff are found, and the red staining of the fibroblasts, etc., with methyl-green pyronin has an entirely different tint from that taken by the cells of Aschoff bodies. The latter stain a deep, rich red and the fibroblasts a bright red, not so deep and with a highly refractile quality, giving them an appearance which for lack of a better term we call translucency. In none of the hearts, although carefully searched for, were bacteria found either in association with the lesions or elsewhere, except in those hearts where vegetative endocarditis was present. In them streptococci were found diffusely scattered in small clumps in the capillaries between normal muscle fibers and also in some of the lesions, but not in all the lesions in these three hearts.

STREPTOCOCCUS RHEUMATICUS AND EPIDEMIC STREPTOCOCCI.

The hearts of the eleven rabbits injected with *Streptococcus rheumaticus* showed changes identical with those described above. One rabbit (No. 13) developed a transient bacteriemia, and at autopsy the only gross lesions in the heart were a few whitish streaks over the endocardium of the left ventricle. This was the only animal injected with an anhemolytic type of streptococcus which developed a bacteriemia in the absence of vegetative endocarditis. Seven rabbits were injected with streptococci from epidemic sore throat and developed the usual pyogenic type of lesions caused by *Streptococcus pyogenes;* they died in the course of a few days. The lesions consisted of extensive degenerative and destructive processes associated with leucocytic infiltration, mainly polymorphonuclear, and with the presence of great numbers of cocci. A few fusiform areas resembling somewhat those described by Jackson were present, but were devoid of giant cells and in no way similar to Aschoff bodies. One rabbit that had been injected with six small non-lethal doses of this streptococcus and lived for ninety-eight days developed multiple arthritis and showed microscopically in the myocardium focalized lesions which occupied a position between the type of lesions produced by *Streptococcus mitis* and that caused by *Streptococcus pyogenes,* but resembling the former more closely.

ILLUSTRATIVE PROTOCOLS.

Rabbit 18.—White, full grown. Dec. 22, 1912. Intravenous injection of one serum glucose agar slant of organism 4,110. Dec. 30. Two slants injected. Jan. 13, 1913. Two slants injected. Jan. 18. Swelling of left fore leg. Feb. 19. Two slants injected. Feb. 20. Dead.

Autopsy.—Heart normal. Diffuse pneumonia of both lungs. Spleen three times normal size; brown in color. Kidneys enlarged. In the right kidney there is a large subcortical hemorrhage. Surface roughened; many depressions surrounded by hemorrhagic zones. One large recent infarct extending from cortex to medulla. Joints normal.

Microscopical Examination. Heart.—Stained with hematoxylin and eosin. The wall of the left ventricle shows a slight grade of diffuse increase of interstitial tissue which in places forms small collections mostly about blood vessels. Near these accumulations of interstitial tissue the muscle fibers surrounded by this tissue stain palely and taper out and are lost in the midst of the connective tissue. A few round cells are present. Other parts show small focalized collections of fibrous tissue, but no diffuse increase in interstitial tissue. Methyl-green pyronin stain gives the same result as above.

Rabbit 36.—White; weight 2,020 gm. Apr. 9, 1913. Intravenous injection of organism 4,110. Apr. 10. Urine normal. Injected one and a half serum glucose agar slants. Apr. 11. Albumin present in urine; few hyaline casts. Apr. 12. Albumin but no casts. Apr. 13. Urine normal. Apr. 16. Trace of albumin, many pus, few red cells. Apr. 18. Injected twenty-four hour tube of blood bouillon culture. Apr. 19. Urine: many pus, and few red cells, no casts. Apr. 27. Three cultures injected. Apr. 29. Urine: albumin, few pus and red cells. Apr. 30. Urine: trace of albumin, few granular casts. Culture: ten drops of blood gave several colonies. May 1. Albumin, few granular and hyaline casts. Culture: 2,000 colonies. May 2. Culture: ten drops of blood gave 4,000 colonies; typical *Streptococcus mitis.* Trace of albumin, moderate number of granular casts, few red cells; clump of pus cells. May 7. Albumin and casts. Culture: six drops of blood gave 5,000 colonies. May 8. No urine in last twenty-four hours. Not eating. Spontaneous twitching and shaking movements. May 9. Poor condition. 11 A. M., 4 c.c. of smoky urine; 12.30 P. M., died. Urine obtained post mortem showed much albumin; large numbers of epithelial casts, and few hyaline and granular casts. Culture gave *B. coli.* Blood culture: 1 c.c. gave innumerable colonies. 5 c.c. of pleural fluid gave 100 colonies; pericardial fluid gave few colonies. Knee joint: 30 colonies from one loop of fluid. Microscopical examination of fluid showed a moderate number of polynuclear cells and a few degenerated forms of streptococci. Endocardiac vegetations and pure cultures of streptococcus.

Autopsy.—Pericardium contains about 1 c.c. of clear serous fluid. Heart dilated; moderate number of minute petechial hemorrhages over the epicardium, along the blood vessels. Tricuspid valve contains diffuse red hemorrhages in

septal leaflet beneath which is a slight projection of the interventricular septum pushing the valve before it. Left auricle: projecting from the mitral ring and practically occluding it are grayish, granular, cauliflower-like vegetations, springing from the entire auricular surface of the mitral flaps to within a short distance of the insertion, but not upon the auricular wall. The vegetations consist of three or four masses, 6 to 7 mm. in diameter, cauliflower-like in appearance, with a dry, finely granular surface. They arise from the auricular surfaces of the flaps; the narrowing of the mitral orifice is caused by the vegetations and not by constriction of the ring. The vegetations pass to the chordæ of the anterior flap as far as the papillary muscle, but none of the chordæ are eroded across. Several minute vegetations affect the anterior papillary muscle near the insertion of the chordæ. The ventricular surface of the anterior flap of the mitral valve is opaque and wrinkled. Left ventricle: the septum just below the aortic valve contains transparent pin-head vegetations about 1 cm. in width. The aortic valve is normal. Chest: each pleural cavity contains 8 to 10 c.c. of serosanguinous fluid. The lungs did not collapse when the chest was opened. On the surface are many bright red areas from 1 to 4 mm. in width beneath which are areas of red hepatization. Peritoneal cavity: slight excess of fluid; parietal and visceral peritoneum show minute petechial hemorrhages. The liver is normal in size and the lobules stand out distinctly, the centers being translucent and the peripheries opaque and fatty. Spleen: the spleen was greatly enlarged, almost black in color, and on the convex surface is a linear scar-like depression, about 1 cm. in length and 2 mm. in depth. The kidneys are normal except for a few subcapsular petechial hemorrhages. The bladder was empty. The large joints contain a small amount of sticky, gelatinous, opalescent material, and few petechial hemorrhages occur in the synovial membranes. The brain shows numerous petechial pial hemorrhages over the convexity of the brain.

Microscopical Examination.—Stained with hematoxylin and eosin. The heart shows slight diffuse leucocytic infiltration as well as large numbers of lesions similar to those described by Bracht and Wächter. The lesions exhibit fragmented nuclei, while the muscle fibers in and about them show an extreme degree of degeneration and fragmentation which are absent from the muscle fibers elsewhere. Some of the lesions are older than others and in them fibroblastic replacement of the muscle fibers has occurred. The methyl-green pyronin stain brings out in addition a few plasma cells. The Gram-Weigert stain discloses streptococci in short chains and small groups in the capillaries between the muscle fibers without reaction about them and not associated with the focal lesions.

Rabbit 13.—White, full grown. Injected with organism 34, *Streptococcus rheumaticus* (Poynton and Paine). Dec. 12, 1912. Received one tube of serum glucose agar culture. Dec. 20. The same. Dec. 26. About twenty-five colonies in ten drops of blood. Dec. 30. Received two tubes of the culture. Jan. 13, 1913. Condition has been good. Received two tubes of culture. Jan. 23. Re-

ceived two tubes of culture. Jan. 29. Received two tubes after passage through seven mice. Feb. 19. Received two tubes. Feb. 24. Limps slightly with left fore leg. Feb. 25. Received two tubes of human blood agar culture. March 1. Received two tubes of human blood agar culture. March 13. The same. March 21. The same. March 24. Suffers from scabies. Heart sounds normal, but animal weak. Etherized.

Autopsy.—A few whitish streaks occur in the endocardium of the left ventricle, and the epicardium is slightly opaque. The organs are normal except that the spleen shows a few minute scars in the capsule.

Microscopical Examination.—Stained with hematoxylin and eosin. Sections of the heart show throughout numerous focalized lesions of round cell infiltration and fibroblastic proliferation with slight atrophy of muscle fibers corresponding typically with those of Bracht and Wächter. Aschoff bodies are absent. There occurs also a slight diffuse round cell infiltration of the myocardium. Methyl-green pyronin stain shows the round cells within the lesions to consist mainly of large mononuclears and to a small extent of small mononuclear and polymorphonuclear cells.

Rabbit 15.—White. Injected with organism 211 (epidemic streptococcus). Dec. 18, 1912. Received one culture on serum glucose agar. Dec. 19. Left hind leg spared. Dec. 20. Limping and ill; coughs. Five drops of blood yield 1,000 colonies. Dec. 21. Moribund; chloroformed.

Autopsy.—Ten drops of blood from heart when plated gave innumerable colonies. Streptococci in pure culture from urine; bile sterile. Heart: grayish white streaks and dots occur in the right ventricle and on its endocardium; hemorrhages occur in the ventricular endocardium beneath the aortic flap of the mitral valve. Lungs: diffuse bronchopneumonia. The kidneys contain focalized purulent streaks in the cortex, and edema of the pelvis and medulla.

Microscopical Examination.—Stained with hematoxylin and eosin. Sections of the wall of the left ventricle show irregular areas, some of which exceed in size the low power field of the microscope, but no areas are fusiform in shape. Within the areas the muscle fibers show various stages of hyaline degeneration, necrosis, and fragmentation, while an edema separates widely the muscle and connective tissue fibers from one another. A moderate leucocytic infiltration, mainly mononuclear and partly polymorphonuclear, is present. Cellular and nuclear debris stain deep blue. In the intercellular and lymph spaces a granular, blue staining material composed of masses of streptococci occurs. Elsewhere in the sections there are slight edema and numerous muscle fibers showing vacuolation and hyaline degeneration. Certain capillaries are filled with

mononuclear leucocytes. The methyl-green pyronin stain brings out in addition some plasma cells in the exudate and in the capillaries. The wall of the right ventricle near the septum shows the same condition and a fibroblastic proliferation about some of the areas of degeneration. The Gram-Weigert stain shows masses of streptococci in the neighborhood of the areas of degeneration and small scattered numbers between the muscle fibers.

DISCUSSION.

From the above it appears that by inoculating rabbits with cultures of *Streptococcus mitis* obtained from cases of subacute bacterial endocarditis, focalized lesions may arise identical with those produced with *Streptococcus rheumaticus* and by Bracht and Wächter with organisms having similar cultural characteristics to the latter. The lesions are entirely different from the submiliary myocardial rheumatic nodules described by Aschoff. The former have neither the structure, location, type of cell, nor staining reactions of the latter. Aschoff bodies consist of rosette, or fan-shaped, collections of large, irregular, often multinuclear cells with rather dense granular protoplasm and vesicular nuclei, arranged about the adventitia of the medium sized arteries or beneath the endocardium, or of fusiform groups of cells lying between the muscle fibers. The cells possess basophilic protoplasm which stains an intense red with methyl-green pyronin. The experimental lesions, however, differ in every way except in being focalized. The lesions in the myocardium of the rabbit are a combination of degenerative and productive processes. Degeneration of the muscle fibers occurs either accompanied by or soon followed by a proliferation of the interstitial tissue which is disproportionate to the extent of the myocardial change. The Aschoff bodies present the character of a productive process in which a specific type of cells with a particular arrangement and location coëxist, and in which the lesion becomes replaced later by fibrous tissue. The lesions described by Jackson and Coombs, while differing from those described by us, differ also from Aschoff bodies, so that further evidence is needed to prove that they are a stage in the development of Aschoff bodies produced experimentally.

We have previously reported finding in the hearts of patients with subacute bacterial endocarditis the same type of lesions as those occurring in our rabbits, which we have called "Bracht and Wächter type of lesions." In several instances the culture inoculated came from these cases. In only two of the hearts were Aschoff bodies found, and in one from a patient giving a history of rheumatism, they were in the healed stage. In the other, a typical verrucose endocarditis and numerous typical Aschoff bodies coëxisted. The Aschoff bodies occurred apart from the Bracht and Wächter type of lesions and no relation between them or transition from one to the other was detected.

Rabbit 29 shows that a streptococcus which when given in lethal doses produces pyogenic myocardial lesions, in sublethal doses administered over a long period of time gives rise to a chronic type of focalized lesion similar to that of Bracht and Wächter.

The fact that *Streptococcus mitis* is not found in the lesions unless vegetative endocarditis occurs coincidentally, indicates that if those organisms are directly responsible for the focalized changes either they disappear early or the lesions result not from the organisms themselves directly but from some toxic product. These possibilities have been considered by Bracht and Wächter and de Vecchi. The former think it probable that the attenuated streptococci are easily destroyed in the body. De Vecchi thinks that if *Streptococcus rheumaticus* is the etiological cause, it should be recognized in all cases.

Since we have demonstrated by cultures *Streptococcus mitis* in inflamed joints of rabbits a considerable time after their localization, it would seem that the streptococci are not more perishable than many other bacteria; and hence we incline towards the view that the focalized myocardial lesions are caused by toxins. It is known that diphtheria toxin causes focalized cardiac lesions; and Loeb and Fleischer have produced similar lesions by means of adrenalin and spartein. The fact that there are a number of substances capable of producing focalized lesions in the myocardium shows definitely that the power to cause degenerative and productive myocardial foci is not a specific property of any one substance or bacterium. Therefore the capacity of *Streptococcus rheumati-*

cus to produce myocardial focalized lesions can be looked upon simply as a property which this organism shares with other bacteria, bacterial and other substances, and is in no way specific. Since the experimental lesions caused by these agents agree in no way with the Aschoff bodies, except as regards the focalized nature, they can not be used as an argument for the etiological relation of *Streptococcus rheumaticus,* or *Streptococcus mitis* (*viridans*), to acute rheumatic fever.

Rosenow claims that the organisms which he has recovered from cases of acute rheumatic fever soon lose in subculture the properties which cause them to produce characteristic lesions in rabbits. The lesions reported above have been produced by various cultures of *Streptococcus mitis,* some recently isolated and others old, and by five strains of *Streptococcus rheumaticus,* all of which were old. We have found the lesions caused by these two types of organisms to be identical. We have not had the opportunity of working with *Streptococcus rheumaticus* in recently isolated cultures.

CONCLUSIONS.

1. By the intravenous injection into rabbits of *Streptococcus mitis,* we have produced focalized myocardial lesions which are identical with those caused by the injection of *Streptococcus rheumaticus,* and with those produced by Bracht and Wächter with *Streptococcus viridans.*

2. The lesions differ from those which we produced by injections of streptococci from the Chicago epidemic of sore throat (epidemic streptococcus).

3. The lesions are not identical with Aschoff bodies and are easily differentiated from them. They also differ from the foci produced by Jackson and Coombs, who describe their lesions as being either Aschoff bodies or similar formations.

4. The myocardial lesions of the rabbit appear to be caused by toxins liberated by the streptococci injected and not by the living organisms themselves.

5. The only point of similarity between the experimental lesions and those found in cases of rheumatic carditis in man is their focalized nature.

BIBLIOGRAPHY.

Achalme, P., *Compt. rend. Soc. de biol.*, 1891, xliii, 651; 1897, xlix, 276.
Andrewes, F. W., and Horder, T. J., *Lancet*, 1906, ii, 708, 775, 852.
Apert, *Compt. rend. Soc. de biol.*, 1898, l, 128.
Aschoff, L., *Verhandl. d. deutsch. path. gesellsch.*, 1904, viii, 46.
Aschoff, L., *Brit. Med. Jour.*, 1906, ii, 1103.
Beaton, R. M., and Walker, E. W. A., *Brit. Med. Jour.*, 1903, i, 237.
Beattie, J. M., *Jour. Path. and Bacteriol.*, 1904, ix, 272; *Edinburgh Med. Jour.*, 1908, xxiii, 391; *Brit. Med. Jour.*, 1904, ii, 1510.
Beattie, J. M., and Yates, A. G., *Jour. Path. and Bacteriol.*, 1912–13, xvii, 538.
Bracht, E., and Wächter, *Deutsch. Arch. f. klin. Med.*, 1909, xcvi, 493.
Coombs, C., *Brit. Med. Jour.*, 1907, ii, 1513; *Quart. Jour. Med.*, 1908–09, ii, 26; *Lancet*, 1909, i, 1377.
Gordon, M. H., *Lancet*, 1905, ii, 1400.
Horder, T. J., *Quart. Jour. Med.*, 1908–09, ii, 289.
Jackson, L., *Jour. Infect. Dis.*, 1912, xi, 243.
Lewis, M. J., and Longcope, W. T., *Am. Jour. Med. Sc.*, 1904, cxxviii, 601.
Leyden, E., *Deutsch. med. Wchnschr.*, 1894, xx, 913.
Libman, E., *Am. Jour. Med. Sc.*, 1912, cxliv, 313; 1913, cxlvi, 625.
Libman, E., and Celler, H. L., *Tr. Assn. Am. Phys.*, 1910, xxv, 5; *Am. Jour. Med. Sc.*, 1910, cxl, 516.
Major, R. H., *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 326.
Menzer, *Berl. klin. Wchnschr.*, 1902, xxxix, 32.
Meyer, F., *Ztschr. f. klin. Med.*, 1902, xlvi, 311; *Deutsch. med. Wchnschr.*, 1901, xxvii, 81.
Poynton, F. J., *Lancet*, 1898, ii, 206; 1899, ii, 1163. Poynton, F. J., and Paine, A., *idem*, 1900, ii, 861, 932, 1136, 1306. Poynton, *International Clinics*, 1904, series 13, ii, 181; iii, 226; *Quart. Jour. Med.*, 1907–08, i, 225; 1909–10, iii, 15. Poynton and Paine, *idem*, 1911–12, v, 463; *Lancet*, 1910, i, 1524. Poynton, F. J., and Shaw, W. V., *Tr. Path. Soc.*, London, 1904, lv, 126.
Pribram, A., Der acute Gelenkrheumatismus, Vienna, 1899.
Rosenow, E. C., *Jour. Infect. Dis.*, 1909, vi, 245; 1910, vii, 411; 1912, xi, 210; *Jour. Am. Med. Assn.*, 1913, lx, 1223.
Sanderson, A., *Guy's Hosp. Rep.*, 1911, lxv, 193.
Schottmüller, H., *München. med. Wchnschr.*, 1910, lvii, 617.
Shaw, *Jour. Path. and Bacteriol.*, 1904, ix, 158.
Singer, G., *Verhandl. d. Cong. f. inn. Med.*, 1897, xv, 116.
Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 417.
Thiroloix, J., *Semaine méd.*, 1897, xvii, 376, 420; *Compt. rend. Soc. de biol.*, 1897, xlix, 268, 882, 945.
Triboulet and Coyon, *Compt. rend. Soc. de biol.*, 1898, v, 124.
de Vecchi, B., *Arch. de méd. exper. et d'anat. path.*, 1912, xxiv, 352.
Walker, E. W. A., *Practitioner*, 1903, xvii, 185.
Walker, E. W. A., and Ryffel, J. H., *Brit. Med. Jour.*, 1903, ii, 659.
Westphal, Wassermann, and Malkoff, *Berl. klin. Wchnschr.*, 1899, xxxvi, 638.

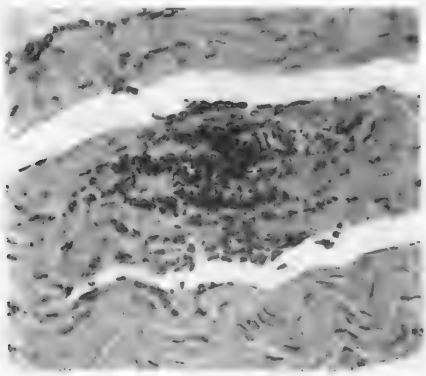


FIG. 1.

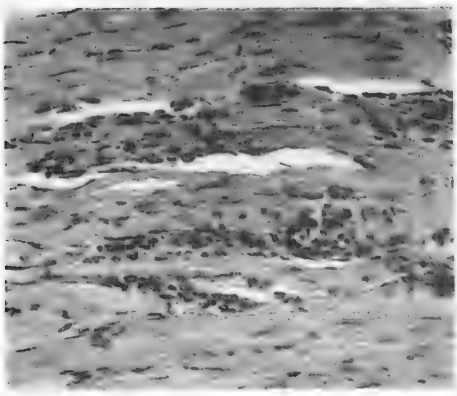


FIG. 2.

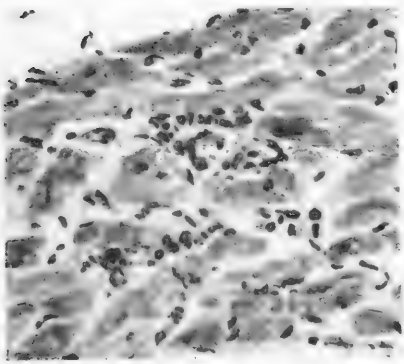


FIG. 3.

(Thalhimer and Rothschild: Focalized Myocardial Lesions.)

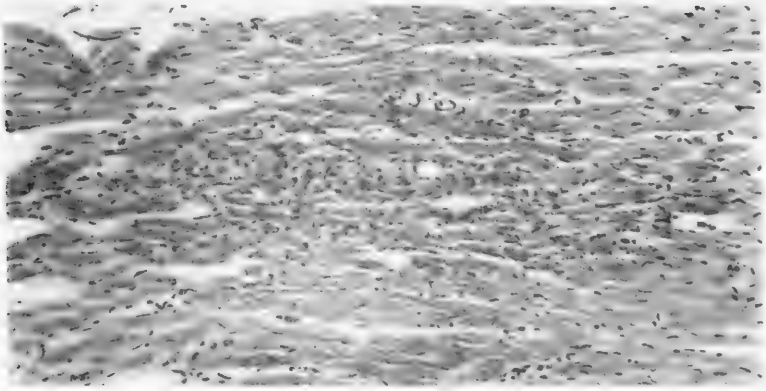


FIG. 4.

FIG. 5.

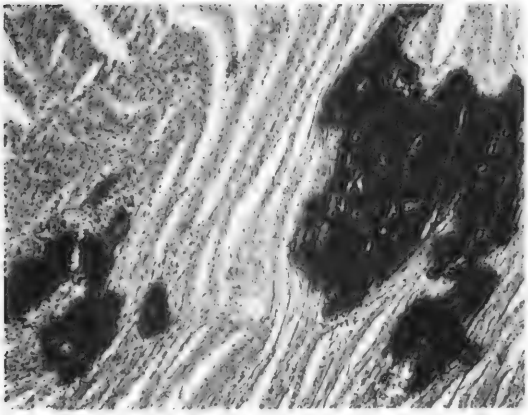


FIG. 6.

(Thalhimer and Rothschild: Focalized Myocardial Lesions.)

EXPLANATION OF PLATES.

PLATE 49.

FIGS. 1 and 2. Earliest stage of focalized myocardial lesion with leucocytic infiltration and beginning degeneration of muscle fibers. Hematoxylin and eosin. × 112.

FIG. 3. Perivascular infiltration, early stage. Hematoxylin and eosin. × 144.

PLATE 50.

FIG. 4. Early stage of focalized myocardial lesion, but more advanced than in figures 1 and 2, showing leucocytic infiltration, degeneration, and fragmentation of muscle fibers and beginning fibroblastic proliferation. Hematoxylin and eosin. × 144.

FIG. 5. Late stage of myocardial lesion showing marked fibrosis of a diffuse type, with here and there leucocytic infiltration. Hematoxylin and eosin. × 48.

FIG. 6. Myocardial lesion showing calcareous infiltration. Hematoxylin and eosin. × 144.

EXPERIMENTAL ARTHRITIS IN THE RABBIT, PRODUCED WITH STREPTOCOCCUS MITIS.*

By M. A. ROTHSCHILD, M.D., and WILLIAM THALHIMER, M.D.
(*From the Pathological Laboratory of the Mount Sinai Hospital, New York.*)

Arthritis has been produced in animals by means of various microorganisms. Those persons who advocate the specificity of *Micrococcus rheumaticus* in acute rheumatic fever find much positive support in the results obtained from the inoculation of animals in which arthritis, endocarditis, or pericarditis occur. In view of this consideration we have been led to inject rabbits with *Streptococcus mitis* isolated *intra vitam* from cases of subacute bacterial endocarditis, with the results to be described in this paper.

This communication does not deal with the etiology of rheumatism. Clinicians and pathologists must first decide what shall be termed acute articular rheumatism. However, as we[1] have pointed out, one group of cases of rheumatism exists in which there is a characteristic myocardial lesion (Aschoff bodies) associated with a verrucous endocarditis and unattended by embolic phenomena. Bacteriological examination in these cases is negative. There is a large second group comprising the bacterial infections of the endocardium, and a third group of bacterial or toxic arthritides with or without endocarditis.

It is possible to produce arthritis in rabbits by means of *Bacillus pyogenes fetidus liquefaciens,* staphylococci, hemolytic streptococci, the streptococcus of epidemic sore throat, Achalme's bacillus, *Micrococcus rheumaticus* isolated from the throat, blood, and joints, *Streptococcus salivarius* and *fecalis* (Horder), the pneumococcus, and *Streptococcus viridans.* Rosenow has reported the production of arthritis with *Streptococcus viridans* in a small percentage, and of endocarditis in a large percentage of rabbits. Our figures have been quite the reverse; that is, 50 per cent. of our rabbits developed

* This work was done under the tenure of a George Blumenthal, Jr., Fellowship. Received for publication, February 3, 1914.

[1] Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 417.

arthritis and 7 per cent. endocarditis. We have employed nine different strains of *Streptococcus mitis* (table I). By reference to the table it will be seen that there is a slight variation in the ability of the different strains to produce arthritis.

TABLE I.

Organism.	No. of rabbits.	Arthritis.	Endocarditis.
4,110	10	7	2
4,147	9	3	0
4,206	7	3	1
4,275	6	5	0
4,170	3	2	0
4,342	3	1	0
4,357	2	1	0
2,524	1	0	0
C	1	0	0
	42	22	3
		50%	7%

CHARACTER OF THE ARTHRITIS.

The rabbits described here are the same as those reported in our communication[2] in which the method of injection, the media used,

TABLE II.[3]

Rabbit 53. Organism 4,275. Injected May 26, 1913. 5 Injections.

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
June 9.....	+[4]	−	+	+
June 14.....	++	−	++	++
June 16.....	++	−	+	++
June 24.....	+++	−	−	+
June 29.....	+++	−	−	+
July 3.....	+++ (Elbow) + (Wrist)	+	−	+
July 7.....	+	−	+	+
July 9.....	+	−	+	+
July 21.....	++	−	−	++
July 28.....	++	−	+	++
Aug. 25.....	−	+	+	+
Sept. 3.....	+ Killed	+	−	+

[2] Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 429.

[3] Beattie (*Jour. Path. and Bacteriol.*, 1910, xiv, 436, table VI) shows in a graphic manner the involvement of the joints of a rabbit that was injected with a streptococcus resembling *Streptococcus rheumaticus*. His table corresponds closely to tables II, III, IV, and V in our series.

[4] In the tables, + = joint affected; − = joint not affected.

and the doses are given. The production of arthritis appears to be independent of the doses of the streptococci and the media employed.

By reference to tables II, III, IV, and V the evanescent and migratory character of the arthritis is seen. Authors have used these characters as an argument for the specificity of a special organism

TABLE III.

Rabbit 10. Organism 4,147. Injected December 10, 1912. 6 Injections. Killed January 24, 1913.

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
Dec. 26	–	–	–	Limps; ankle swollen.
Dec. 27	–	–	–	No limp; ankle swollen.
Dec. 28	–	–	–	–
Dec. 29	–	–	–	Ankle swollen.
Dec. 31	–	–	–	Ankle swollen.
Jan. 6	–	–	–	Ankle swollen.
Jan. 13	–	–	–	Swelling smaller.
Jan. 17	–	–	–	Swelling smaller.
Jan. 22	–	–	–	Swelling smaller.
Jan. 24	–			Swelling smaller.

TABLE IV.

Rabbit 18. Organism 4,110. Injected December 22, 1912. 4 Injections. Died February 20, 1913.

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
Dec. 25	–	–	Limps	–
Dec. 27	–	–	Ankle swollen	–
Dec. 31	–	Stiff	Stiff	–
Jan. 13	–	–	–	–
Jan. 18	Limps; ankle swollen	–	–	–
Jan. 25	–	–	–	–

TABLE V.

Rabbit 70. Organism 4,357. Injected June 24, 1913. 2 Injections. Died July 13, 1913.

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
June 27	–	–	Stiff	Stiff
June 28	–	–	+	+
June 29	–	–	+	+
July 3	–	–	Knee +	Knee +
July 7	–	–	+	++
July 9	–	–	+	+
July 13	–	–	–	–

in causing acute articular rheumatism. The fact that the joints after extensive involvement return to a normal condition has been emphasized by Poynton and Paine, Beattie, and others. The arthritis produced by *Streptococcus mitis* has the same general characteristics. In some of the animals a chronic type of arthritis arises, the joint cartilages being eroded, the articular surfaces proliferated, and a partial ankylosis of the joint resulting. Poynton and Paine have seen the same results follow from *Micrococcus rheumaticus.* For control experiments we injected a number of rabbits with streptococci obtained from the sore throat epidemic in Chicago. Our results agreed with those of Davis who produced arthritis in every animal inoculated with organisms from the Chicago and Boston epidemics of sore throat. We injected a series of rabbits with hemolytic streptococci, and our results agreed with those of Cole who states: "In most cases the pathological changes in the joints are mild, consisting mainly of edema about the joint, the fluid becoming turbid, sticky, and tenacious, injection of the capsule and slight infiltration, mainly of the villi, with polymorphonuclear leucocytes." The arthritides which developed in our series were acute in their development, almost purulent in nature, and accompanied by some destruction of the surfaces of the articular cartilages. A series of rabbits (eleven) was injected separately with five different strains of *Streptococcus rheumaticus,* one strain isolated by Poynton and Paine, one by Beattie, one by Lintz, and two by Rosenow. 45 per cent. of the animals developed an arthritis; and the character of the arthritis was identical with that caused by *Streptococcus mitis.*

CHARACTER OF THE JOINT EXUDATE.

The exudate was of the same character as that described by the observers working with *Streptococcus rheumaticus.* It was of a thick, sticky, gelatinous consistence and contained many large endothelial cells with distinct phagocytic properties, a moderate number of polymorphonuclear leucocytes, and a large quantity of homogeneous material. The exudate is both articular and periarticular, having a decided tendency to extend down the tendon sheaths, and in one rabbit it extended from the knee joint to the ankle. In only

one instance was there any tendency towards decided pus formation.

Bacteriological studies of the exudate (table VI) show that we were able to demonstrate microörganisms in a comparatively small

TABLE VI.

Organism.	No. of rabbit.	Blood cultures.	Joints.	
			Smears.	Cultures.
4,147	1	0	0	+
4,147	10	0	0	0
4,142	42	0	0	+
4,170	27	Not taken	0	0
4,170	43	0	0	0
4,357	70	0	0	0
4,110	18	0	0	0
4,110	36	+	+	+
4,110	37	0	0	0
4,110	38	+	0	0
4,110	47	0	0	0
4,110	48	0	+	0
4,110	49	Autopsy records lost		
4,206	39	0	+	0
4,206	57	0	0	0
4,206	59	+	+	+
4,275	35	0	+	+
4,275	41	0	0	0
4,275	51	0	0	0
4,275	52	0	0	0
4,275	53	0	+	0
4,342	55	0	+	0

percentage. In smears the organisms are almost always found intracellularly both in the large endothelial cells and in the polymorphonuclear leucocytes. The organisms can be recovered in cultures in only about one third of the animals. In one rabbit with a positive blood culture both smears and cultures from the joint were negative. Two of the rabbits having a vegetative endocarditis with positive blood cultures gave cultures from the joints. In the table bacteriological studies of the joints which have subsequently returned to normal are included; consequently the figures may not give a true index of the relative finding of streptococci in the affected joints. Meyer claims as one of the characteristics of the arthritis produced by *Streptococcus rheumaticus* that organisms can not be recovered from the joints. Other observers, however, have isolated the organism in the joints of cases of rheumatism and in arthritis produced experimentally in animals.

CONCLUSIONS.

1. We have produced arthritis in 50 per cent. of the rabbits injected with *Streptococcus mitis.*

2. The character of the arthritis is identical with that produced by *Micrococcus rheumaticus.*

3. The exudate in and about the joints partakes of the same nature as that caused by *Streptococcus rheumaticus.*

4. Bacteriological studies show that *Streptococcus mitis* can be recovered from about one third of the affected joints.

5. Arthritis produced by other types of streptococci differs by reason of greater destruction of tissue, by being more permanent in character, and by the exudate containing large numbers of polymorphonuclear leucocytes.

6. The deduction of a distinct variety or species of streptococcus based upon the power to cause arthritis in rabbits is unwarranted.

BIBLIOGRAPHY.

Beattie, J. M., *Jour. Path. and Bacteriol.*, 1904, ix, 272; *Edinburgh Med. Jour.*, 1908, xxiii, 391; *Brit. Med. Jour.*, 1904, ii, 1510.

Cole, R., *Jour. Infect. Dis.*, 1904, i, 714.

Davis, D. J., *Jour. Am. Med. Assn.*, 1912, lviii, 1283.

Davis, D. J., and Rosenow, E. C., *ibid.*, p. 773.

Horder, T. J., *Quart. Jour. Med.*, 1908–09, ii, 289.

Lintz, W., *Jour. Am. Med. Assn.*, 1912, lviii, 615.

Meyer, *Deutsch. med. Wchnschr.*, 1901, xxvii, 81.

Poynton, F. J., *Lancet*, 1898, ii, 206; 1899, ii, 1163. Poynton, F. J., and Paine, A., *idem*, 1900, ii, 861, 932, 1136, 1306; *International Clinics*, 1904, series 13, ii, 181; iii, 226. Poynton, F. J., and Shaw, W. V., *Tr. Path. Soc.*, London, 1904, lv, 126. Poynton, F. J., *Quart. Jour. Med.*, 1907–08, i, 225; 1909–10, iii, 15; 1911–12, v, 463; *Lancet*, 1910, i, 1524.

Rosenow, E. C., *Jour. Am. Med. Assn.*, 1913, lx, 1223.

A STAGE IN THE MIGRATION OF THE ADULT TERTIAN MALARIAL PARASITE. EVIDENCE OF THE EXTRACELLULAR RELATION OF THE PARASITE TO THE RED CORPUSCLE.*

By MARY ROWLEY-LAWSON, M.D.

(*From the Laboratory of Dr. Mary Rowley-Lawson, New London.*)

Plates 51 to 55.

In previous publications[1] I have stated that I believe the malarial parasite to be extracellular and to migrate from corpuscle to corpuscle. To recapitulate:

I believe the malarial parasite to be extracellular throughout its existence; that is, except for brief periods when it is free in the blood serum, it is attached to the external surface of the red corpuscle.

The parasite attaches itself to the red corpuscle by means of delicate pseudopodia thrown out from the cytoplasm of the parasite. It encircles and squeezes up into a mound a portion of the hemoglobin (figures 1 to 17); thus it maintains its position on the outside of the red corpuscle.

From my observations I believe that each parasite in the course of its development, destroys several red corpuscles, migrating from one to another, thus giving a rational explanation of the anemia which occurs in the course of the malarial infections.

The evidence in favor of migration is as follows: 1. The great destruction of red corpuscles, which is usually out of all proportion to the number of parasites present in the peripheral blood, provided that each parasite destroys but one corpuscle. 2. Instances of multiple infection of red corpuscles by several young parasites,

* Aided by a grant from The Rockefeller Institute for Medical Research. Read before the Society of American Bacteriologists, Montreal, January 2, 1914. Received for publication, February 21, 1914.

[1] Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420; *Jour. Exper. Med.*, 1913, xvii, 324.

sometimes as many as seven. They cannot all grow on one corpuscle, and even if conjugation were amongst the possibilities, it would not account for odd numbers nor for infection of a corpuscle by parasites of varying stages of development. Therefore if these parasites are not to die, they must migrate. 3. What appear to be stages in parasitic migrations, which may be summarized as follows: (a) Pigmented parasites free in the blood serum. They may be seen in various stages of development, compact, ring-shaped, ameboid, and with protoplasmic pseudopodia, the pigmentation being evidence of previous attachments to red corpuscles. (b) Pigmented parasites in various stages of development, attached to red corpuscles the hemoglobin of which is apparently unaltered. Here, as in stage (a), the pigmentation is evidence of one or more previous attachments. (c) Pigmented parasites in various stages of development on decolorized red corpuscles and on corpuscular skeletons. (d) Pigmented parasites partly on and partly off degenerated red corpuscles, caught apparently in the act of abandoning them. (e) Corpuscular skeletons, which are the expanded, dehemoglobinized remains of red corpuscles, usually more or less semilunar in shape, granular, and staining a delicate pink. These skeletons are most frequently seen free from parasites.

This paper is intended principally as a description of stage (d), but it illustrates also the extracellular relation of the adult tertian parasite to the red corpuscle. In one specimen of blood from a series of smears taken consecutively, one after another, from a patient who had taken no quinine, I found over 100 thirty (?) hour parasites partly on and partly off expanded red corpuscles showing granular degeneration (figures 19 to 54, and 59 to 78). I have occasionally seen parasites partly off red corpuscles in other specimens; but never so many in any one smear as in this case. Free parasites, in the same stage of development as the parasites seen partly off the red corpuscles, were found in the same smear (figure 55). I believe that these parasites were preparing to migrate, for the infected red corpuscles, though retaining in general their contour, were undoubtedly degenerated,[2] being more or less dehemo-

[2] Segmenting parasites on red corpuscles showing similar granular degeneration (figures 56 to 58) were found in the same smear.

globinized. Finding so many parasites in similar stages of development in this phase, in one smear, seems to me good evidence in favor of migration as against a condition caused by technique. I can think of no evidence in favor of technique that may be responsible for the phenomenon. The smear was made on a slide, with the blood spread only in one direction, yet the parasites were seen reaching away from the corpuscles in various directions. If rough smearing was the cause of the parasites being partly off the corpuscles, the parasites would be drawn off from the corpuscles all in one direction. And technique would not account for a condition where the corpuscle is distorted exactly at right angles to the direction in which the parasite reaches out from the corpuscle (figures 22 and 27).

In many instances that portion of the parasite which is off the red corpuscle, may be seen resting along the periphery of the infected cell (figures 20, 31, 32, 34 to 37, and 39), which would not be the case were technique responsible for its dislocation.

That the adult parasite is attached to the external surface of the red corpuscle and not submerged beneath its surface seems to me to be proved by figures 1 to 58.

Parasites developed beyond the young ring-form stage (figures 1 to 11) may occasionally be seen to be attached to fairly healthy appearing red corpuscles, the bodies of the parasites resting on the periphery of the corpuscles (figures 13 to 16). These parasites are certainly not submerged beneath the surface of the red corpuscles, but have, I believe, only recently attached themselves, and are therefore coming rather than going. Occasionally a segmenting body may be seen extending beyond the periphery of a red corpuscle with unbroken contour (figure 18).

As to the parasites that are preparing to migrate (figures 19 to 54), to the trained eye those that are on the upper surface of the granular degenerated corpuscles can easily be differentiated from the parasites on the under surface; i. e., in figure 22, the parasite is partly off the upper surface of the red corpuscle, while in figure 33 the parasite is seen to be partly off the under surface of the corpuscle.

The unbroken contour of the majority of the infected red cor-

puscles showing portions of parasites extending beyond the periphery, as well as appearance of the adjacent cells, with the exception of a in figure 36, would seem to preclude any suggestion of unusual violence in the spreading of the smear. I use the adjective unusual, as it seems to me that it would require a very unusual violence to produce a condition as rare as that seen in the accompanying plates. I believe the infected red corpuscles were already too much damaged by the action of the parasites to regain their contour on the slide if the parasites had been forcibly squeezed or pulled out of them in spreading the smear, especially parasites of such advanced growth. I do not believe that in smearing the blood it would ever be possible to reach the parasite, if it were submerged beneath the surface of the red corpuscle, in order to pull or squeeze it out, without damaging that corpuscle beyond repair; for instance, in figure 20 the body of the parasite rests on the periphery of the corpuscle and only short pseudopodia are seen to be attached to the corpuscle, the contour of which is unbroken; and in figure 19 only a slender process extends beyond the periphery of a corpuscle with unbroken contour. If these parasites were submerged beneath the surface of the red corpuscles, could technique have produced the condition? I think not.

Healthy appearing red corpuscles may be damaged by technique when the blood is being smeared and not regain their contour. The healthy, uninfected, but distorted, red corpuscle seen at a in figure 35 did not regain its shape after being injured by obvious technique. In malarial infections, especially in the æstivo-autumnal infections I have seen many red corpuscles which had not regained their normal contour after having been damaged by the parasites while in the circulating blood.[3]

If the young parasite fastens itself to the external surface of a red corpuscle and proceeds with the destruction of the corpuscle while it is so attached (see young parasites encircling corpuscular mounds on the decolorized red corpuscles in figures 10 and 11), why should the adult parasite follow any other procedure? Indeed, peripheral mounds may be seen in connection with adult parasites

[3] An article describing and illustrating these damaged red corpuscles will appear later.

(figure 17, a). And why should the adult parasite assume a more or less characteristic ring-form unless it be for the purpose of securing its attachment to the surface of the red corpuscle? For the ring shape, in connection with the adult parasite, may be explained, as it is explained in connection with the young parasite, as a result of the parasite encircling and drawing up into a mound a portion of the hemoglobin substance of the red corpuscle in order to secure its attachment to the surface of the corpuscle.

In fresh blood preparations, parasites may be seen to abandon red corpuscles which have not been entirely destroyed. Laveran,[4] in referring to the external relation of the malarial parasite to the red corpuscle, says: "Osler noticed that the amoeboid bodies which adhere to the red blood corpuscles can be detached and become free in the blood," adding, that this was one of the arguments on which he relied in maintaining that the parasites were only attached to the surface of the red corpuscles. Marchiafava and Bignami[5] and Monacho and Panichi[6] have stated that quinine may cause certain parasites to abandon the red corpuscles. This being so, if the parasites were submerged beneath the surface of the red corpuscles, then the quinine would have to destroy the substance of the red corpuscles in order to release the parasites; but with the parasites attached to the external surface of the corpuscles, their detachment is more easily explained.

SUMMARY.

1. What appear to be certain definite stages in the migration of the malarial parasite from red corpuscle to red corpuscle may be demonstrated by thorough and persistent observations,—not minutes spent on each specimen, but many hours.

2. The migration of the malarial parasite from red corpuscle to red corpuscle gives a reasonable explanation of the loss of red corpuscles which cannot be accounted for by the destruction of the infected corpuscles at the time the parasites segment.

3. Migration to other red corpuscles is a satisfactory explana-

[4] Laveran, A., Paludism, translated by Martin, J. W., London, 1893, 41.

[5] Marchiafava, E., and Bignami, A., in Twentieth Century Practice of Medicine, New York, 1900, xix, 461.

[6] Monacho and Panichi, cited by Ewing, J., Clinical Pathology of the Blood, 2d edition, New York and Philadelphia, 1903, 458.

tion of the ultimate fate of the young parasites seen in instances of multiple infection of single corpuscles.

4. In the light of the facts here presented, it would seem impossible to explain the instances of the parasites partly on and partly off degenerated red corpuscles as the result of technique. A stage in the migration of the parasite seems to me to be the probable interpretation of the phenomenon.

5. A corpuscular mound encircled by an adult parasite, when seen at the periphery of the red corpuscle, should have the same significance and interpretation in reference to the extracellular relation of the parasite to the corpuscle, that it has when it is seen in connection with a young parasite.

6. The adult ring-form parasite should have the same interpretation as the young ring-form parasite.

7. Attachment to the external surface of the red corpuscles seems to me to be the only possible interpretation of the appearances of the parasites pictured in this article.

EXPLANATION OF PLATES.

PLATE 51.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIGS. 1 to 3. Young parasites attached to the external surface of the red corpuscles. Those attached to peripheral mounds may be seen at a; the other parasites are attached to surface mounds.

FIG. 4. The nucleus of the young parasite attached to the peripheral corpuscular mound at a is distorted by technique.

FIG. 5. A young parasite attached to a peripheral corpuscular mound at a, which is almost entirely dehemoglobinized.

FIG. 6. A young parasite attached to the red corpuscle. The body of the parasite rests on the periphery of the corpuscle.

FIG. 7. A young parasite attached to the periphery of a peripheral corpuscular mound. The mound is almost entirely dehemoglobinized.

FIGS. 8 and 9. Young parasites encircling peripheral corpuscular mounds.

FIG. 10. A young parasite encircling a surface corpuscular mound; the entire corpuscle is much dehemoglobinized.

FIG. 11. A young parasite encircling a peripheral corpuscular mound. The corpuscle is much dehemoglobinized.

FIG. 12. A pigmented parasite attached to the surface of a red corpuscle. The mounds to which it is attached can be seen at a.

FIG. 13. A pigmented parasite attached to the surface of a red corpuscle, the hemoglobin of which appears to be unaltered. As a large part of the parasite

rests on the periphery of this corpuscle, I believe that the parasite has only recently attached itself to it, hence the unaltered appearance of the hemoglobin.

FIG. 14. A parasite attached to the external surface of the corpuscle. The body of the parasite is seen to be off the corpuscle, having been dislocated by technique.

FIG. 15. A pigmented parasite on the periphery of the red corpuscle, the appearance of the pseudopodia suggesting that the parasite is starting to encircle a corpuscular mound. The slightly degenerated appearance of the infected corpuscle is probably due to the action of the young parasite.

FIG. 16. A pigmented parasite, the body of which rests on the periphery of the corpuscle. This parasite is attached to the under surface of the red corpuscle. There is another parasite attached to the upper surface.

FIG. 17. An adult pigmented parasite attached to the external surface of the red corpuscle. The peripheral mound to which it is attached can be seen at a.

FIG. 18. Young parasites resulting from a very recent segmentation; the red corpuscle has not been entirely destroyed. One of the segments can be seen to be partly off, and two entirely off the corpuscle at a.

FIG. 19. An adult pigmented parasite on the upper surface of a granular degenerated red corpuscle. A pigmented protoplasmic process arising from the parasite can be seen extending beyond the periphery of the corpuscle at a.

FIG. 20. An adult parasite resting on the periphery of a granular degenerated red corpuscle. With the exception of three short and delicate pseudopodia, the parasite is free from attachment to the corpuscle.

FIG. 21. An adult parasite on the upper surface of a degenerated red corpuscle, with a portion of its protoplasm extending beyond the periphery of the infected corpuscle.

PLATE 52.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIG. 22. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. Note that the parasite is lying off the corpuscle at right angles to the way the cell is distorted.

FIG. 23. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. The mound to which the parasite is attached shows less of the granular degeneration than the rest of the corpuscle.

FIG. 24. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. The surface mound to which the parasite is attached is almost entirely decolorized by the action of the parasite. This mound can be seen at a.

FIGS. 25 and 26. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. The mound to which the parasite in figure 26 is attached can be seen at a.

FIG. 27. An adult parasite partly off the under surface of a degenerated red corpuscle. The granular outline of the infected corpuscle can be traced across the upper surface of the parasite between o and o. The mound to which the parasite is attached can be seen at a. Note that the distortion of the corpuscle is at right angles to the way the parasite lies off the corpuscle.

FIGS. 28, 29, and 30. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. The mounds of attachment of the parasite in figure 30 can be seen at a.

FIGS. 31, 32, and 33. Adult parasites partly on and partly off the under surfaces of degenerated red corpuscles. In figures 31 and 32, the part of the parasite that is off the corpuscle can be seen resting on the periphery of the infected corpuscles.

PLATE 53.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIG. 34. An adult parasite partly on and partly off a degenerated red corpuscle; the part of the parasite that is off the corpuscle may be seen resting on the periphery of the corpuscle.

FIG. 35. An adult parasite partly on and partly off the upper surface of a red corpuscle which is less dehemoglobinized than the other corpuscles shown which are infected by parasites partly off and partly on. At a is a red corpuscle that has been injured by technique and has not regained its normal shape.

FIGS. 36 to 39. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. In figures 36, 37, and 39 the part of the parasite which is off the corpuscles can be seen resting on the periphery of the infected corpuscles. In figure 37 a young parasite is seen attached to the infected corpuscle.

FIG. 40. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle. Another adult parasite can be seen on the under surface of the infected corpuscle.

FIGS. 41 and 42. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 43. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle. The mound of attachment is seen at a.

FIGS. 44 and 45. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. In figure 44 a young parasite is seen attached to the infected corpuscle. The mound to which the adult parasite is attached can be seen at a.

PLATE 54.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIG. 46. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle.

FIGS. 47, 48, and 49. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 50. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle.

FIGS. 51, 52, 53, and 54. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 55. An example of the free parasites found in the same smear with, and in the same stage of development as, the parasites seen partly on and partly off the degenerated red corpuscles.

FIGS. 56, 57, and 58. Segmenting parasites on corpuscles which are in a similar stage of degeneration to many of the corpuscles which show the parasites partly off them, and which came from the same smear.

PLATE 55.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

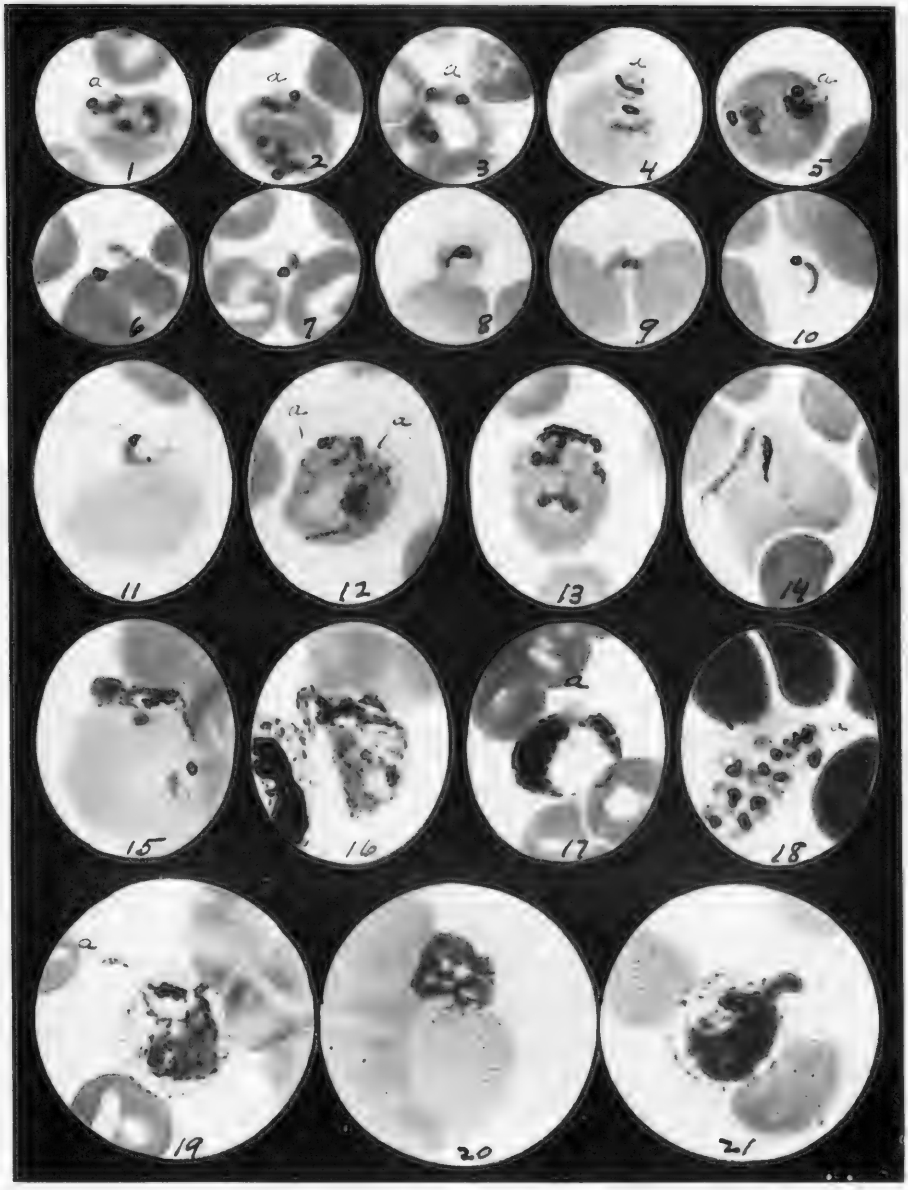
FIG. 59. (Corresponds to figure 18.) A segmenting parasite showing some of the separate segments beyond the periphery of the infected red corpuscle.

FIG. 60. Two young parasites attached to the surface of a red corpuscle; the body of one of the parasites is on the periphery of the corpuscle with the attaching pseudopodium arranged in the form of a loop overlying the corpuscular substance; the other parasite is attached to a decolorized surface mound.

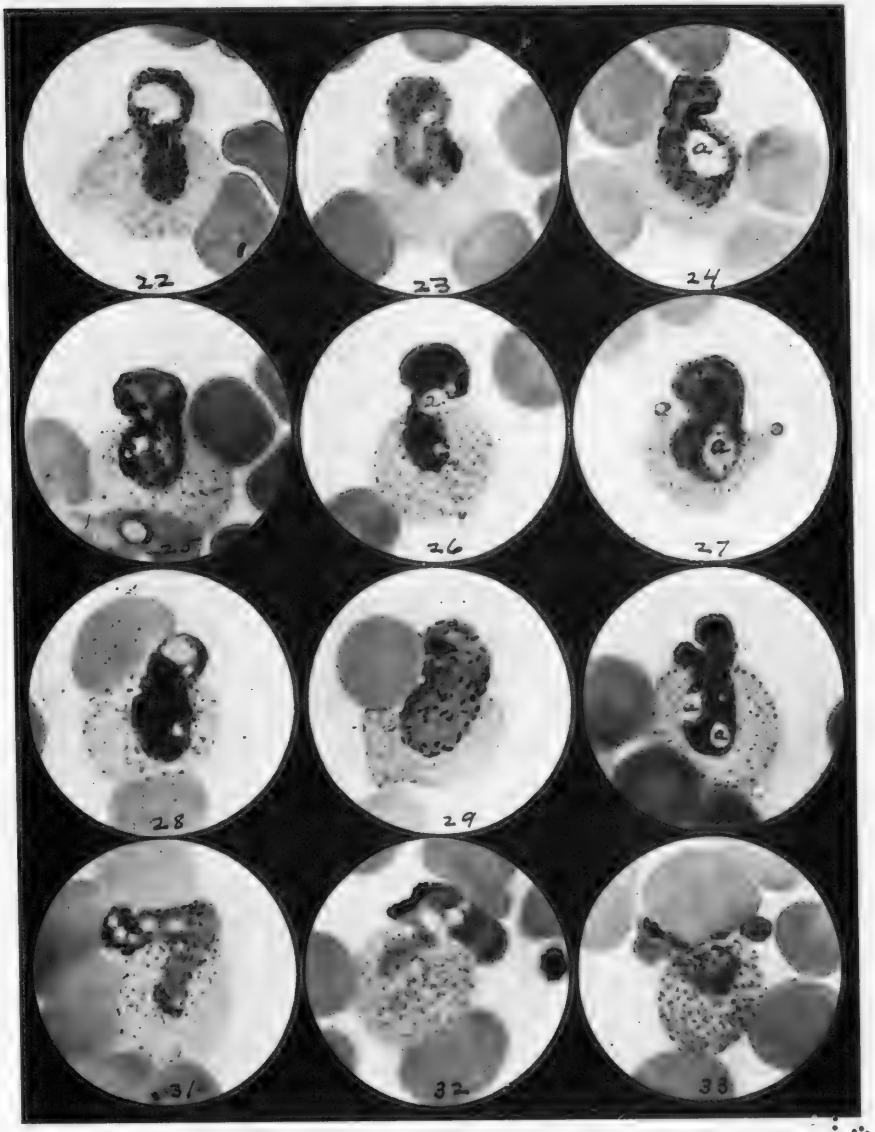
FIG. 61. (Corresponds to figure 12.) A pigmented parasite attached to the surface of a red corpuscle. The mounds to which the parasite is attached can be seen.

FIG. 62. (Corresponds to figure 15.) A pigmented parasite on the periphery of a red corpuscle, with the pseudopodia overlying the corpuscular substance.

FIGS. 63 to 78. (Correspond to figures 25, 28, 23, 24, 37, 26, 30, 22, 29, 33, 32, 31, 21, 20, 19, and 36.) Adult pigmented parasites partly on and partly off granular degenerated red corpuscles. Some of the parasites may be seen to be on the upper surfaces of the corpuscles, others on the under surfaces. In figure 18, with the exception of three short and delicate pseudopodia, the parasite is free from attachment to the corpuscle. In figure 19 only a delicate pseudopodium extends beyond the periphery of a red corpuscle with unbroken contour.



(Lawson: Migration of the Adult Tertian Malarial Parasite.)



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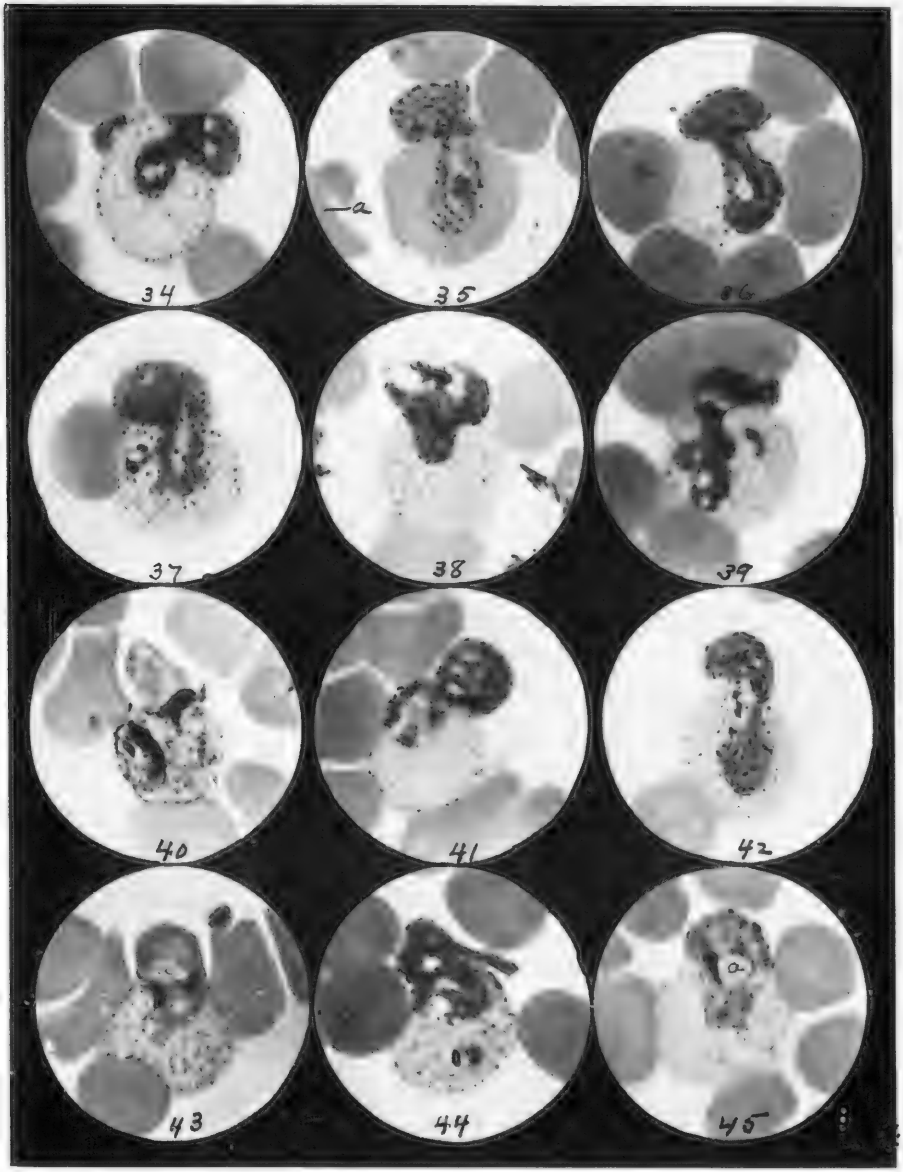

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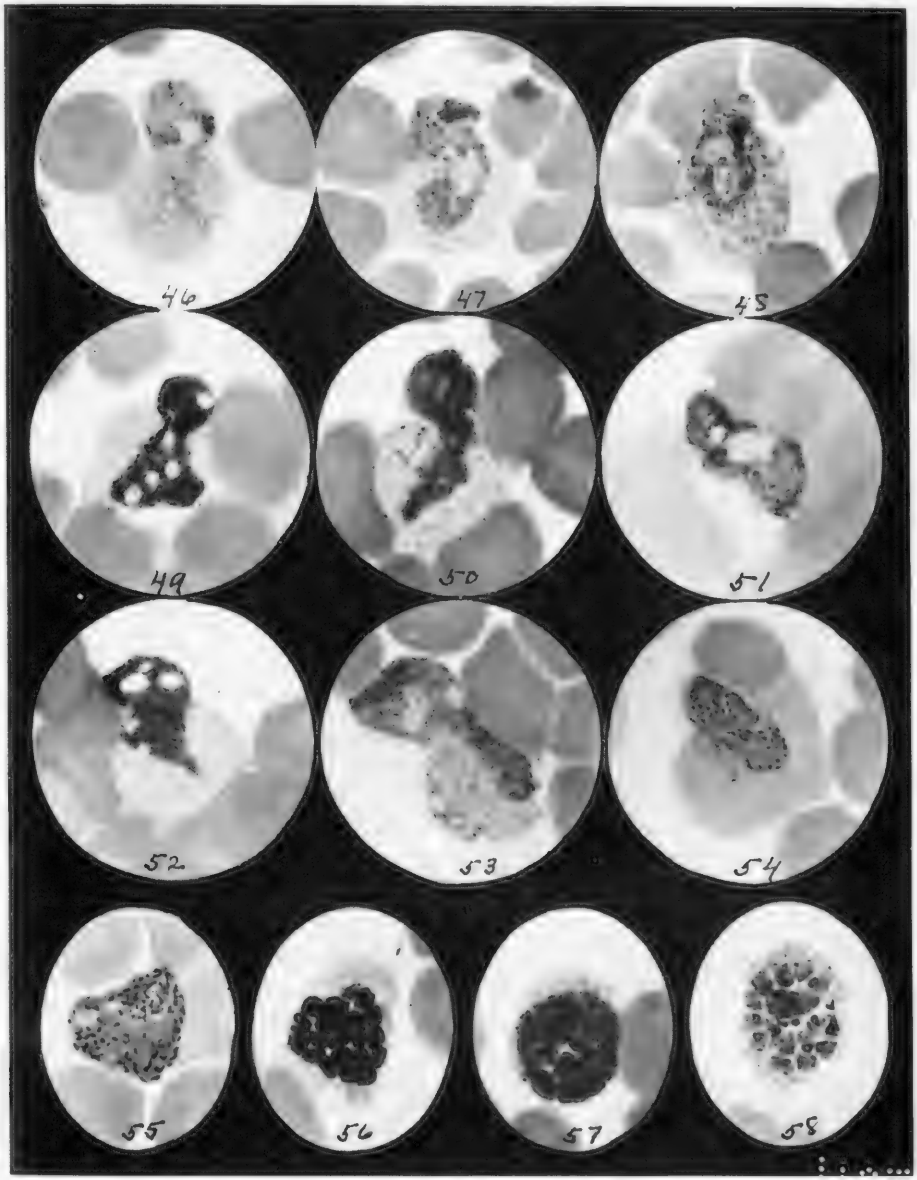

(Lawson: Migration of the Adult Tertian Malarial Parasite.)

(Lawson: Migration of the Adult Tertian Malarial Parasite.)

THE NATURE OF SERUM ANTITRYPSIN.

Studies on Ferment Action. XIII.*

By JAMES W. JOBLING, M.D., and WILLIAM PETERSEN, M.D.

(*From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.*)

The efforts that have been made to determine the nature of the ferment-inhibiting substances of the blood give no definite information concerning their significance or their character. At one time it was thought that the antitryptic index of the serum would prove to be a valuable aid in the diagnosis of cancer.

Brieger and Trebing (1) stated that 90 per cent. of the patients suffering from carcinoma or sarcoma, whom they had examined, showed an increase of antitrypsin in the blood. Von Bergmann and Meyer (2) confirmed this observation, though they also found a similar increase in 24 per cent. of non-cancerous patients. Recent work indicates that the increased ferment-inhibiting action of the serum cannot be relied upon as a diagnostic test for cancer. It is frequently present in the acute infections, such as pneumonia, typhoid fever, etc.; in chronic infections, such as tuberculosis and syphilis; in Graves' disease; and in severe anemias. The action of the serum has been ascribed to chemical constituents and to specific immune bodies which act as antiferments. Meyer (3) believes that the antiferment is a true antibody, and that the ferments of the tissue cells act as antigens. He concludes that trypsin and leucoprotease are not so important in this respect. Eisner (4) and Wiens (5) also believe that the antiferment is a true antibody, but that the antigen is the ferment liberated by the polymorphonuclear cells. On the other hand, Jürgensen (6) found no relation between the antitryptic index and leucocytosis. Halpern (7) inoculated dogs with the pancreas of dogs, and found an increase in antitrypsin, but no increase in antipepsin. Eisner (4) studied the inhibiting action of serum against various ferments, and concluded that it possessed a special affinity for trypsin. Some writers have asserted that the serum is more active against trypsin of the same species, but Weil (8) and others have disproved this.

Morgenroth (9) thought that he obtained a specific antirenin by inoculating animals with renin, while Achalme (10) states that he produced a specific antitrypsin by inoculating animals with trypsin. Other investigators repeated the work of Morgenroth and Achalme and have failed to confirm their results. Hamburger (11) showed that sodium chloride destroys pepsin in a neutral solution, thereby invalidating the evidence that a specific antipepsin is present in the blood; immune bodies are active only in a neutral or slightly alkaline reac-

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tion. We must conclude, then, that sufficient proof has not been advanced to support the view that the ferment-inhibiting substances of the blood are true antibodies.

Hedin (12) has shown that charcoal will bind trypsin and that the binding is proportional to the quantity of charcoal and to the time of interaction. Strong acids and alkalies quickly destroy trypsin, but Chittenden and Cummins (13) found it to be much more resistant to these agents in the presence of protein. Kudo (14) states that all the mineral acids are active in this respect, sulphuric acid being active in a dilution of one in a thousand, while the organic acids are much less so. Bayliss (15) and Abderhalden and Gigon (16) found that some of the products of tryptic activity possess the property of inhibiting the action of the ferment, the free amino acids being more active than the polypeptids.

Schwartz (17) concluded that the antitryptic activity of the serum is due to the lipoids. He found that he could partially inactivate sera by extracting them with ether, and that the sera could be reactivated by the addition of lecithin. Sugimoto (18) observed a decrease in the antitryptic strength of sera after extraction with ether and benzol, and concluded that the lipoids were the active constituents. He believes that a complete extraction of the fat, and the dissociation of the lipoid-protein combinations will cause a removal of the ferment-inhibiting action. He also tested alcoholic extracts which were prepared from egg-white, beef brain, and beef liver, and found them inactive as anti-enzymes when used as emulsions in solutions of sodium chloride. Meyer (3) suggests that the effect of the ether was due to the destruction of the anti-enzymes and not to their extraction. He also refers to the normal antitryptic index found in the lipoid-rich sera of luetics and diabetics. He found that it was not destroyed by extracting the dried serum with ether, and the fluid serum with petroleum ether, but states that this does not disprove the possibility of its being a lipoid-protein combination. He does not believe that protein cleavage products are the inhibiting agents. Cobliner (19) assumes that lipoids are not the active agents, as dried sera extracted with ether, chloroform, or petroleum ether do not lose their antitryptic activity. He found that the inhibiting action was increased if the serum was shaken with olive oil. Kirchheim (20) concludes that the antiferment merely prolongs the action of the trypsin and does not destroy it. He observed that chloroform reduced the inhibiting action of the serum.

Rosenthal (21) believes that the action of the serum is due to protein cleavage products, and that its increase in carcinoma and other diseases with cachexia can be thus explained.

Two years ago we observed that sera preserved with chloroform soon lost their antitryptic action. The same observation had been made previously by Delezenne and Pozerski (22), but we did not know of it. This discovery convinced us that the ferment-inhibiting substances of the serum were lipoids, and that they were soluble in fat solvents. We (23) have published the results of some preliminary work based on this idea, in which it was shown that the soaps of the unsaturated fatty acids obtained from various sources possessed the property of acting as anti-enzymes. These results caused us to study the ferment-inhibiting action of the blood to see if it was due to the same agents.

The Fuld-Gross technique was used by most of the investigators engaged in the study of the anti-enzymes of the blood. We have already discussed in

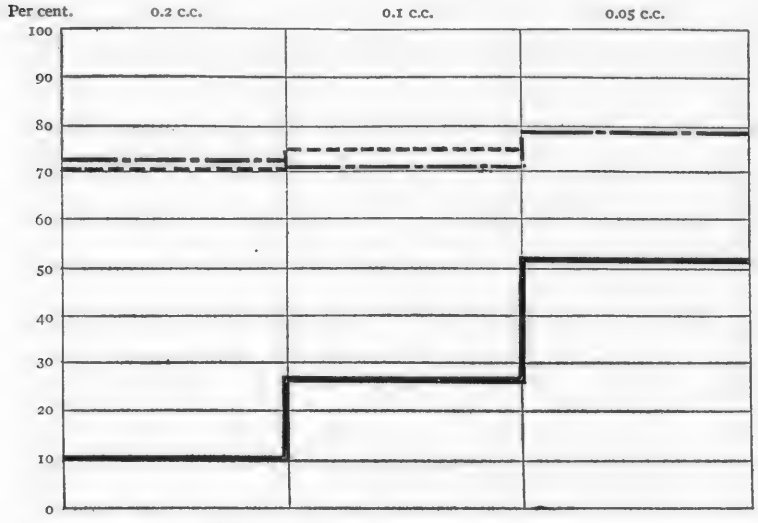
another paper (23) the difficulties we encountered in the use of this method. In this work, as well as in that previously reported, our interpretations were based upon the total incoagulable nitrogen determined according to the method recommended by Folin.

INFLUENCE OF CHLOROFORM OR ETHER EXTRACTION ON SERUM ANTITRYPSIN.

Considerable work has been done with lipoid solvents in order to show the relation which exists between the lipoids of the serum and antitrypsin, but with contradictory results. In most of the work the solvent was allowed to act for a short time only, and, in some instances, on the dried serum. In the first experiment we wished to determine the influence of chloroform or ether on the enzyme-inhibiting action of the serum. Fresh dog serum was diluted 1 to 5 with salt solution, and divided into three parts. To one part was added chloroform; to another ether; the remaining portion was preserved without the addition of any agent. The three flasks containing the sera were then kept at room temperature for forty-eight hours. The mixtures were shaken frequently during this time. Before testing, the excess of chloroform or ether was removed with pipettes, and the serum then filtered through filter paper until clear. They were mixed in various dilutions with trypsin and placed in the incubator for thirty minutes. Casein was then added and the mixture incubated for two hours at 37° C. A control flask containing only trypsin and casein was incubated for the same period of time. The lines on text-figure 1 show the amount of digestion in each flask as compared with that obtained in the control tube which contained no serum. This method will be followed in all the subsequent experiments.

Text-figure 1 shows the results of extracting the serum with ether or chloroform. The lines in the text-figure show that in the tube containing 0.2 of a cubic centimeter of the unextracted serum, the digestion was only 10 per cent. of that obtained in the control, while in the tubes containing the extracted serum, the digestion was 70 per cent. of the control. In other words, the chloroform and ether had removed 60 per cent. of the inhibiting action of the serum. Dog serum has not the high anti-enzyme power of human serum, and this accounts for the comparatively large amount

used. This experiment has been repeated many times with human serum, horse serum, dog serum, and guinea pig serum, and similar results have been obtained with each. It was found necessary to shake the mixtures of chloroform and ether frequently, otherwise the inhibiting action of the serum was not removed. At least twice as much solvent as serum should be used.



Black line = original serum.
Dotted line = ether-extracted serum.
Broken line = chloroform-extracted serum.

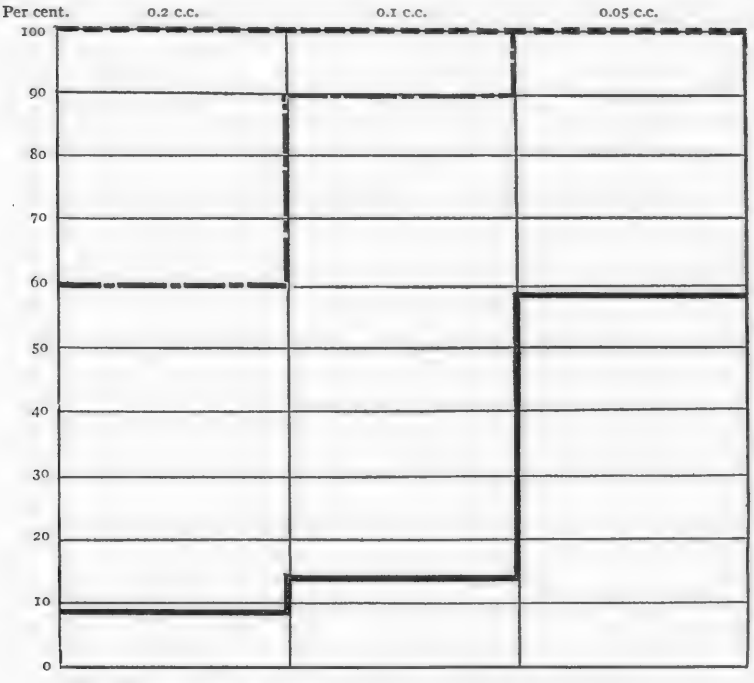
TEXT-FIG. 1. Effect of ether or chloroform extraction on serum antitrypsin.

Two possibilities must be considered in explaining the action of chloroform or ether on the enzyme-inhibiting action of the serum: first, that the substances causing the inhibiting action have been destroyed; and, second, that they have been taken up by the solvents.

INFLUENCE OF THE REACTION ON EXTRACTED SERA.

The next experiment was conducted to see if the activity of the serum could be restored by rendering it neutral or slightly alkaline in reaction. In order to duplicate the conditions present in the last experiment, ether was added to the serum in two tubes, and the

mixtures were kept at room temperature. A third tube of serum was kept without any preservative. After four days the ether was evaporated from one tube, the serum shaken thoroughly, and then made slightly alkaline with a dilute solution of sodium hydrate. This was done to see if the acids could be saponified, and the activity



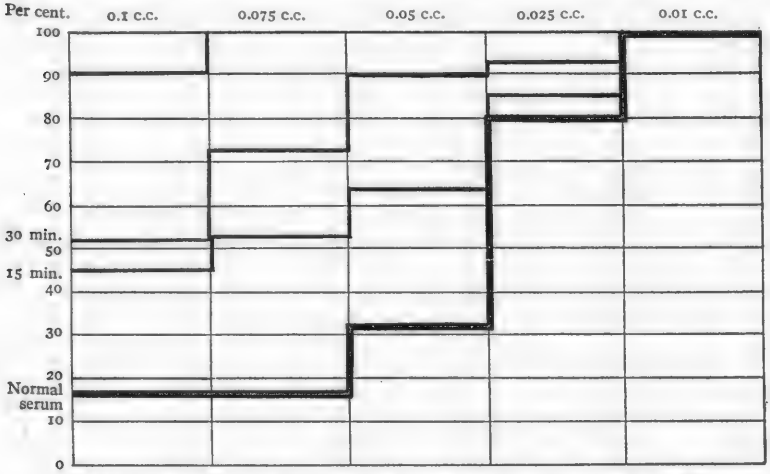
Black line = human serum.
Dotted line = ether-extracted human serum.
Broken line = ether-extracted human serum, ether evaporated.

TEXT-FIG. 2. Effect of evaporating ether extract and partial reactivation of serum.

of the serum restored. The ether was removed from the second tube by means of a pipette, and the serum was then filtered several times through filter paper. It was made neutral by adding a dilute solution of sodium hydrate. Text-figure 2 shows the results obtained in this experiment. The chart shows that only 8 per cent.

of digestion was obtained in the flask containing 0.2 of a cubic centimeter of the untreated serum, while 100 per cent. of digestion was obtained in the flask containing the same amount of ether-extracted serum. The serum which had been made slightly alkaline after evaporation of the ether showed about half the inhibiting action possessed by the one to which nothing had been added. These results indicate that the solvent does not destroy the anti-enzyme.

We must assume that the action of the alkali is due to a combination with some substance soluble in ether, as the alkali did not re-



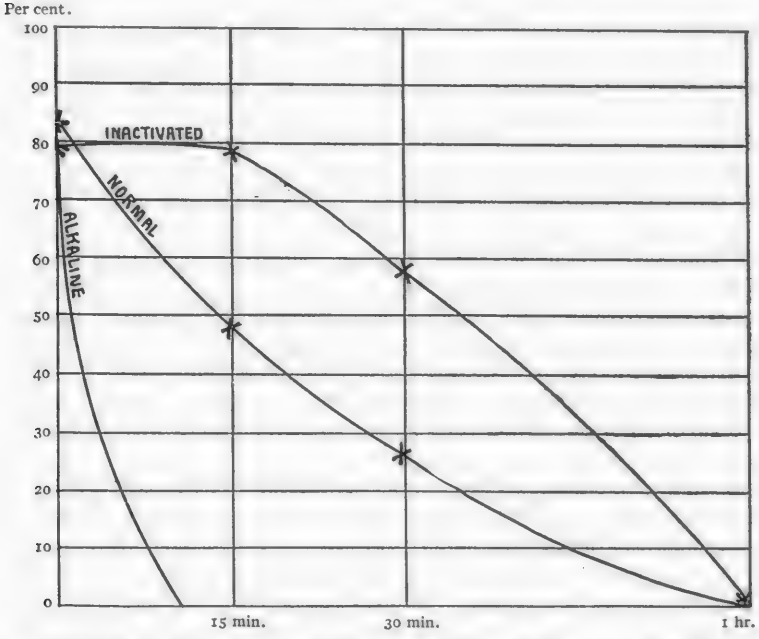
TEXT-FIG. 3. Effect of chloroform on serum antitrypsin at 37° C.

store the activity of the serum from which the ether had been removed, but partially restored it to the serum from which the ether was evaporated. Under these conditions the alkali would not be likely to combine with lipoids such as lecithin, cholesterol, etc., and so only the fatty acids remain. It cannot be expected that the full strength of the inhibiting action observed in the untreated serum should be obtained here. Following the evaporation of the ether, the extractives would be more or less insoluble in the serum and this would render it impossible to saponify all the free fatty acids without the aid of heat and an excess of alkali. If the removal of the inhibiting action at room temperature by chloroform is aided by the action of the serum lipase, a shorter time should be required

by incubating the serum at 37° C., ferments being more active at this temperature.

TIME REQUIRED FOR CHLOROFORM EXTRACTION AT 37° C.

In the next experiment we wished to determine the length of time required for chloroform to remove the ferment-inhibiting action of the serum when the mixture was kept at 37° C. Chloroform was added to fresh serum, thoroughly shaken, and the



TEXT-FIG. 4. Rate of removal of serum antitrypsin in normal, inactivated, and alkalinized serum at 37° C.

mixture was placed in the incubator at 37° C. At fifteen minute intervals some of the serum was removed and tested for its ferment-inhibiting properties. In separating the serum from the chloroform it is necessary to centrifuge the emulsion at high speed for about five or ten minutes, and then to filter it through coarse filter paper several times until the serum is quite clear and all chloroform evapo-

rated. This experiment demonstrates that the ferment-inhibiting action of the serum is almost wholly lost after sixty minutes' incubation with chloroform (text-figure 3).

In the next experiment we wished to determine if lipase was essential to the removal of the inhibiting substances by the chloroform. The serum was divided into three portions. One portion was heated at 54° C. for thirty minutes in order to destroy the lipase; the second portion was made distinctly alkaline in order to prevent the serum from becoming acid; and the third portion was used as a control. Chloroform was added to all three portions and they were then placed in the incubator. A portion of each lot was removed at fifteen minute intervals and tested against trypsin for its anti-enzyme properties (text-figure 4).

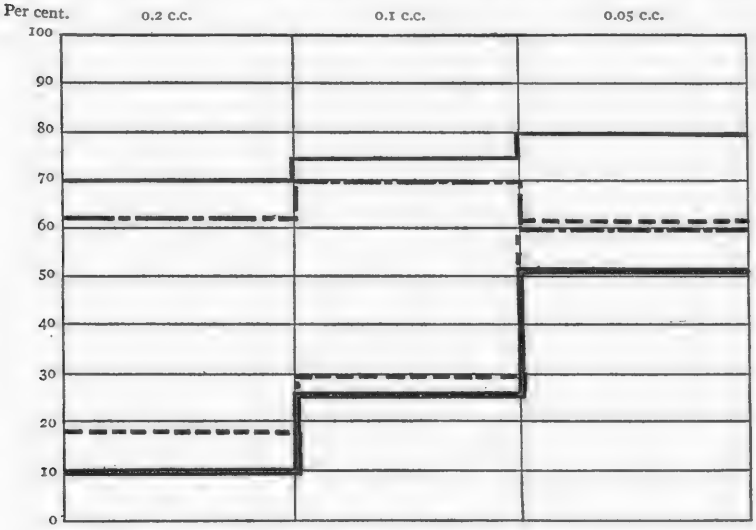
The results show that lipase is not essential to the removal of the inhibiting substances by the chloroform, and indicate that the inhibiting substances are probably fatty acid esters which are soluble in chloroform and ether. There was very little decrease in the inhibiting action of the inactivated serum during the first fifteen minutes of incubation. This may have been due to the absence of the lipase, or to some physical change in the serum caused by heat.

FATTY ACIDS AS ANTIFERMENTS.

The results obtained in the previous experiments indicate that the inhibiting agents are compounds of the fatty acids, and the next experiment was planned to see if this was the case.

Ether was added to fresh dog serum and the mixture was allowed to stand for two days at room temperature. The ether was then removed and divided into two portions. One portion was made slightly alkaline with sodium alcoholate and evaporated to dryness. The soaps were then dissolved in water and filtered; the acids liberated with hydrochloric acid, taken up in ether, and resaponified. The second portion of ether was shaken with water and the ether then evaporated at low temperatures. The soaps prepared from the first portion, the emulsion prepared from the second, and the serum from which the ether extracts were obtained, were tested for their ferment-inhibiting action. The original untreated serum was used

as a control. Text-figure 5 shows the percentage of casein digested by trypsin after it had been incubated for thirty minutes with these preparations. The lines on the chart show that the soaps prepared from the ether extract cause nearly as much inhibition as the untreated serum, while the water emulsion gives 62 per cent. of diges-



Heavy black line = original serum.
Lighter black line = extracted serum.
Dotted line = ether extract saponified.
Broken line = ether extract emulsified.

TEXT-FIG. 5. The antitryptic effect of the ether extract of normal serum when saponified.

tion. The inhibition caused by the emulsion is probably due to some of the acids combining with the alkali which had been used in the preparation of the casein solution. The soaps thus formed would of course inhibit ferment action. The original untreated serum gave 10 per cent. of digestion, and that extracted with ether 70 per cent. Complete removal of the inhibiting action of the serum is rarely obtained in less than four days' extraction at room temperature, and then, as a rule, only with chloroform.

Up to the present time all observers have failed to secure any

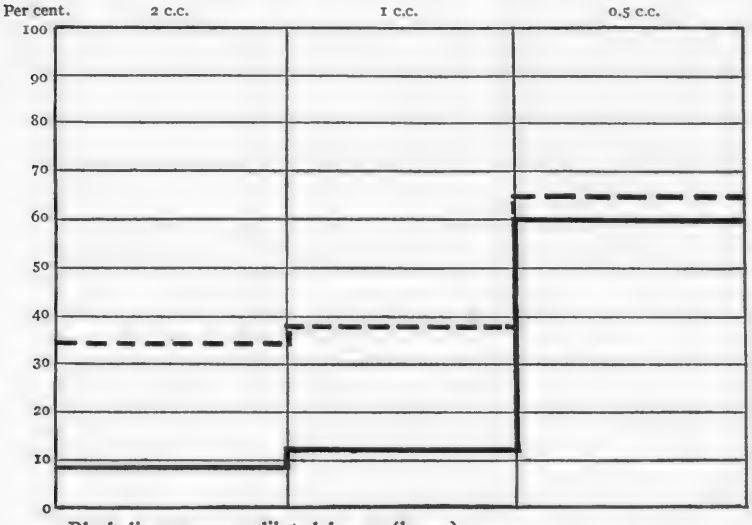
antitryptic effect of the ether or chloroform extracts. This is due solely to the fact that in the simple emulsions with which they worked, the intimate contact of the trypsin and the unsaturated fatty acid complex, which is the basis of the antitryptic activity, is not brought about. In the native serum these lipoids are in a state of dispersion sufficiently great to permit such contact, and we can reproduce it only by saponifying these fatty acid radicals and thus bringing them into a colloidal solution again. We have found that we can at all times secure the antitryptic effect in almost its original value from the extracts, if care is taken to secure complete saponification. If such extracts are not well saponified, so that droplets of unsaponified material can be observed under the high power microscope, then either little or none of the antitryptic effect will be recovered. The results of this experiment indicate that the inhibiting action of the serum is due to the fatty acids. It is not necessary to assume that they can act only as soaps; probably the esters containing unsaturated fatty acids are just as effective.

REMOVAL OF ANTIFERMENT BY FILTERING ACID SERUM THROUGH KAOLIN.

The results that we have been discussing were probably due to the extraction of the fatty acids by the solvent. The fatty acids can also be liberated by means of other acids, in which case they should be thrown out of solution and removed by filtration. The next experiment was planned to find out if the inhibiting substances could be removed by filtering through kaolin serum which had been made strongly acid with hydrochloric acid.

Twenty cubic centimeters of fresh dog serum were diluted with forty cubic centimeters of water, and five cubic centimeters of N/10 hydrochloric acid were added. The mixture was shaken thoroughly, allowed to stand for a few minutes, and then filtered several times through kaolin. After standing for a short time to permit all the serum to drain off, the kaolin was heated with sodium alcoholate. The mixture was then filtered and the filtrate evaporated to dryness. The soaps were dissolved in water and tested for their ferment-inhibiting properties. The acid serum obtained after filtration was made neutral and also tested. Some of the original unacidified

serum was filtered through kaolin to see if kaolin filtration would exert any influence on normal serum. Text-figure 6 shows the results of filtering acidified serum through kaolin. The inhibiting action of the serum which was made acid and then filtered through kaolin was completely lost, and is therefore not shown in the chart. The soaps prepared from the kaolin were active as inhibiting agents

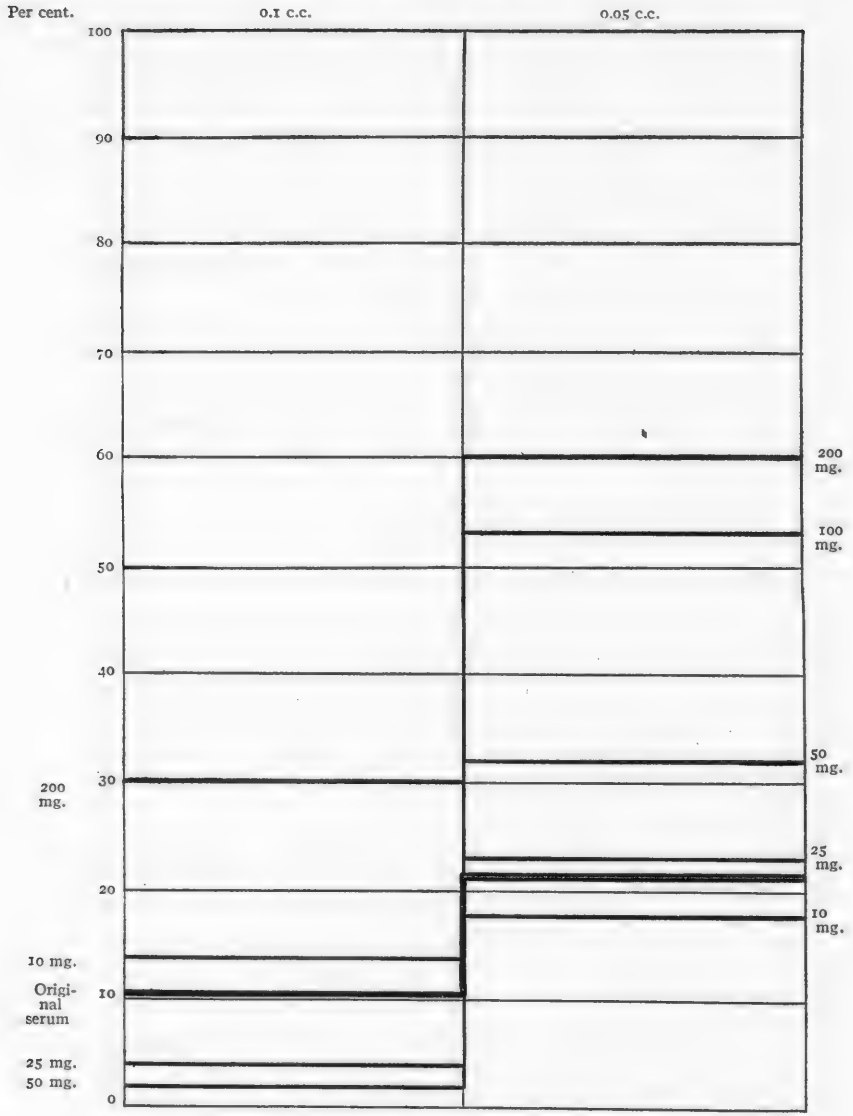


Black line = serum diluted by 10 (horse).
Dotted line = kaolin extract saponified, *i. e.*, $\frac{1}{10}$ strength of serum.
TEXT-FIG. 6. Antitryptic effect of kaolin extract saponified.

and indicate that the fatty acids are the active substances present in the serum. The strength of the soap solution was about 0.1 of that of the untreated serum. We have not been able by this method to recover a larger amount of the inhibiting substances, though the serum has usually lost all its anti-enzyme properties.

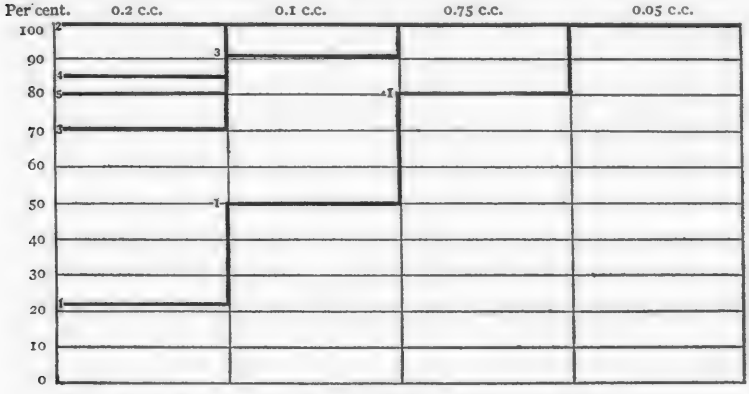
INFLUENCE OF IODIN OR POTASSIUM IODIDE ON ANTIFERMENT.

In our previous work (23) we have shown that soaps of the unsaturated fatty acids lose their ferment-inhibiting properties when treated with iodin. For this purpose we used iodin dissolved in a solution of potassium iodide and removed the excess of iodin by



TEXT-FIG. 7. Effect of varying amounts of potassium iodide on serum antitrypsin.

shaking the mixture with chloroform. This last step was necessary, as iodin inhibits tryptic activity. In the present work we were unable to use the iodin-iodide of potassium mixture, as we have shown that serum loses some of its ferment-inhibiting properties even when shaken with chloroform for a short time. For these reasons we have first investigated the action of potassium iodide alone on the ferment-inhibiting action of the serum.

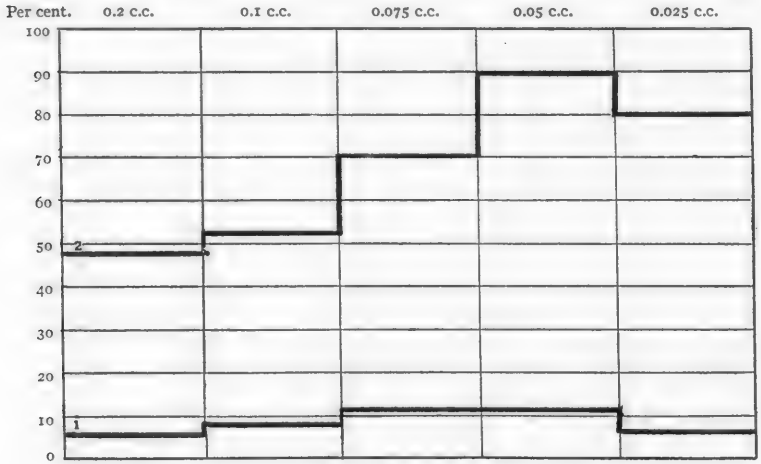


1 = dog serum, 3 days at room temperature.
2 = 1 c.c. dog serum + 200 mg. potassium iodide, 3 days at room temperature.
3 = 1 c.c. dog serum + 100 mg. potassium iodide, 3 days at room temperature.
4 = 1 c.c. dog serum + 200 mg. sodium iodide, 3 days at room temperature.
5 = 1 c.c. dog serum + 100 mg. sodium iodide, 3 days at room temperature.

TEXT-FIG. 8. Effect of sodium iodide and potassium iodide on antitrypsin *in vitro*.

Fresh human serum was divided into six portions of five cubic centimeters each. To one of these portions, potassium iodide was added in the proportion of 10 milligrams to one cubic centimeter; to the second, 25 milligrams to one cubic centimeter; and to the third, 50 milligrams; to the fourth, 100 milligrams; and to the fifth, 200 milligrams. The sixth received no potassium iodide and was used as a control. The tubes containing the sera were placed in the incubator for three hours and afterwards kept at room temperature over night. The following morning they were incubated for thirty minutes with trypsin, and the casein was then added. Control tubes containing trypsin and potassium iodide, without serum, showed

that the chemical did not inhibit the activity of the trypsin. Text-figure 7 shows the influence of potassium iodide on the ferment-inhibiting action of the blood. The chart shows that potassium iodide even under these conditions destroys some of the ferment-inhibiting action of the serum. In the first column in which 0.1 of a cubic centimeter was used, the influence was not marked; but in the second column in which 0.05 of a cubic centimeter of serum was used, there was considerable diminution of the inhibiting action.



1 = guinea pig serum (1 c.c. diluted by 10), normal.
2 = guinea pig serum (1 c.c. + 200 mg. potassium iodide), over night in incubator.

TEXT-FIG. 9. Effect of potassium iodide on antitrypsin of guinea pig serum.

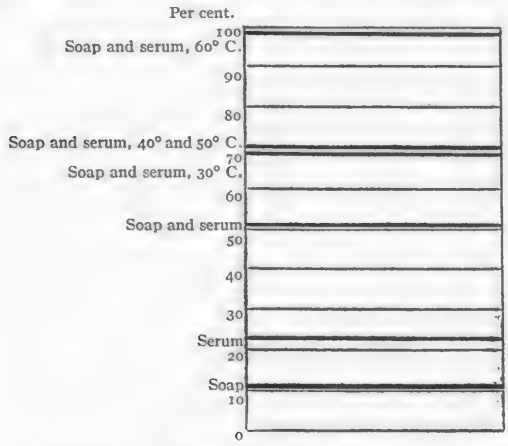
Another experiment was made to see if other iodides would act in a similar manner. In this instance fresh dog serum was treated with sodium iodide and potassium iodide. The technique used was the same as that described for the preceding experiment except that the mixtures were kept for two days at room temperature. The results of this experiment (text-figure 8) are more striking than those of the preceding one. This may be due to the fact that the iodides were in contact with the serum twenty-four hours longer. The sodium iodide is just as active as the potassium iodide in removing the inhibiting action of the serum.

Still more conclusive are the results obtained with guinea pig serum, as this serum is more active as an inhibiting agent. The mixture of serum and potassium iodide stood in the incubator over night. Text-figure 9 shows the influence of potassium iodide on guinea pig serum.

The effect of potassium iodide on the serum antitrypsin seems to be most marked on fresh sera. Treatment of serum with an equal volume of strong solution of hydrogen peroxide, and incubated over night, also causes complete loss of its antitryptic power.

INFLUENCE OF HEAT.

Serum loses its ferment-inhibiting properties if it is heated for thirty minutes at 65° C. If the fatty acids are the inhibiting agents, they must undergo some physical or chemical change which renders them inactive. In the next experiment we wished to determine the



TEXT-FIG. 10. Effect of heat on the antitryptic activity of soap and serum. 1 c.c. contains 0.1 c.c. of serum and 0.01 gm. of soap.

influence of heat on the ferment-inhibiting properties of soap when it is mixed with serum. We had found that soaps dissolved in water did not lose their activity when heated at 100° C., and so it was of interest to determine if they were more susceptible in the presence of serum protein.

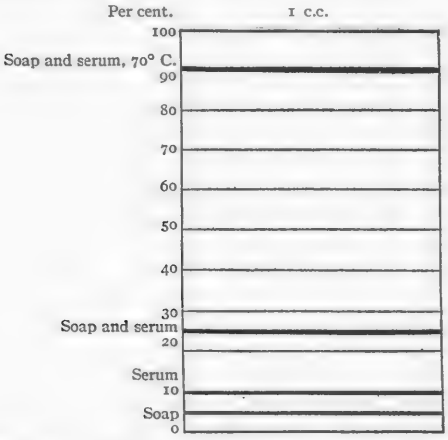
Linseed oil soap, with an iodin value of 110, was used in this experiment. A 10 per cent. solution of dog serum was prepared and to this was added an equal amount of a 1 per cent. soap solution. Portions of the mixture were heated for thirty minutes at 30°, 40°, 50°, and 60° C., and then tested for their ferment-inhibiting properties. The unheated mixture of soap and serum and the unheated soap and serum solutions were tested separately as controls (text-figure 10). The chart shows that the soap solution was active and permitted only 12 per cent. of digestion, while the serum was less active, permitting 23 per cent. of digestion. The unheated mixture of soap and serum gave 52 per cent. of digestion, while the mixtures heated at 30°, 40°, and 50° C. gave 70 per cent. In the mixture heated at 60° C. the inhibition of ferment action was completely lost.

The amount of inhibition obtained with the unheated mixture of soap and serum was less than that obtained with the soap or the serum alone. This indicated that some of the inhibiting action was destroyed by mixing the serum and soap. In our previous work we found that a slight degree of acidity was sufficient to destroy the activity of the soaps and so we immediately tested the serum used in this experiment. It was found that one cubic centimeter of the serum required 0.1 of a cubic centimeter of an N/10 sodium hydrate solution to neutralize it. This explains why the mixture of soap and serum was less active than the soap or serum separately. The high degree of acidity was probably due to the fact that the serum was several weeks old.

Experiments were then conducted to determine the influence of heat on soap mixed with neutral serum. The same acid serum was made slightly alkaline to phenolphthalein by means of a sodium hydrate solution. Text-figure 11 shows the influence of heat on linseed oil soap when mixed with neutral serum.

Here again the unheated mixture of soap and serum does not inhibit the action of trypsin as much as the soap or the serum alone, though it does show more inhibition than when the serum was acid in reaction. It is possible that here, also, there was some dissociation of the soaps which caused a decrease of inhibiting action. Heating at 70° C. caused almost complete loss of activity of the soap-serum mixture.

Further experiments were made with serum which had been rendered distinctly alkaline by adding 0.1 of a cubic centimeter of N/10 sodium hydrate solution to the neutral serum. This alkaline serum-soap mixture lost only 30 per cent. of its inhibiting proper-



TEXT-FIG. 11. Effect of heat on the antitryptic activity of soap and serum in neutral reaction.

ties when heated for thirty minutes at 70° C. In other words, loss of activity as the result of heating appears to bear some relation to the reaction of the serum.

DISCUSSION.

The results obtained in the preceding experiments justify, we think, the conclusion that the ferment-inhibiting properties of the serum are due to the presence of compounds of the unsaturated fatty acids. Their solubility in ether or chloroform suggests that they are not in the form of soaps, but are present as esters. In some experiments which are not described in this paper, we found that a fine emulsion of olein in serum inactivated by heat, or in salt solution, possessed some ferment-inhibiting action, the inhibiting action bearing a distinct relation to the degree of dispersion. This observation is significant as numerous investigators have reported that the antitrypsin of the serum is increased after eating.

In our study of the ferment-inhibiting substances present in tuberculous caseous material (24) we at first thought that the unsaturated fatty acids were present in the form of soaps, but subsequent experiments showed that they could also be extracted by means of chloroform or ether, after which the material was digestible by trypsin. These results indicate that here also the acids in the form of esters are active as inhibiting agents.

If fresh serum is tested daily for its ferment-inhibiting action it will be found that its strength decreases from 5 to 10 per cent. for the first few days; after that it remains almost constant for an indefinite period of time. With this decrease in anti-enzyme activity there is an increase in acidity. The acidity is probably due to the liberation of fatty acids by the action of the serum lipase on the fatty acid compounds present. If serum lipase is mixed with ethylbutyrate and then incubated, the degree of acidity will be found to be practically the same at the end of four hours as after twenty-four hours. But if the mixture is made neutral at the end of the first four hours and again incubated, the acidity at the end of the second four hours will be almost equal to that obtained after the first incubation. In other words, the ferment can act to a certain point only; after that its action is inhibited either by the amount of acids liberated or by the synthetic compounds formed from the acids. When chloroform or ether is added to the serum, the acids are taken up by the solvent almost as fast as they are liberated, and so they do not interfere with the action of the lipase. Under these conditions the ferment remains active until most of the substrate consisting of the fatty acid compounds has been destroyed. We have found that chloroform acts better than ether in removing the inhibiting agent, and this is to be expected as chloroform is known to exert less influence on ferment activity. This hypothesis also explains why a serum can be left for months with other preservatives without losing all its antiferment properties. In order to remove the inhibiting substances with chloroform it is necessary that a large amount of the solvent be used, and that the mixture be shaken frequently.

Two possibilities must be considered in explaining the destruction of the ferment-inhibiting properties of the serum by heat: first,

it might be due to saturation of the unsaturated fatty acids; second, to the mechanical inclusion of the fatty acid compounds in the fine coagula caused by the heating. If the fatty acids become saturated when heated with protein the combination formed must be easily dissociated, as their activity is not permanently lost. If an inactive soap-serum mixture is saponified with strong solutions of sodium hydrate, and the fatty acids are then liberated in the usual manner and resaponified, it will be found that the soaps are just as active as those obtained from an unheated portion of the same mixture. At first we thought the destruction of the inhibiting action was due to the mechanical inclusion of the fatty acid compounds in the fine coagula caused by heating, but subsequent experiments with sera made strongly alkaline with sodium hydrate disproved this. Under these conditions, coagula would not form, yet the resistance of the inhibiting substances to heat was greatly increased. We have noticed frequently that there is an increase of from 5 to 20 per cent. in the antiferment activity of sera heated for fifteen minutes at 54° to 55° C., especially in sera that are lipemic.

Thiele (25) found that the neutral fat of chyle was adsorbed by serum when the mixture was kept for several hours at 37° C. The fat in such a mixture could not be extracted with ether, but the combination could be broken up by peptic digestion and by treatment with alcohol. A similar explanation may apply to our observation that a soap-serum mixture heated at 70° C. loses its antienzyme properties. The iodin values of the heated and unheated mixtures are the same. We must therefore assume that the combination formed is one which is readily dissociated.

The effect of the potassium iodide is probably due to the liberation of small amounts of free iodin which can then saturate the unsaturated carbon bonds. Thus the iodin value (determined by precipitating the serum proteins by alcohol and titrating the alcoholic filtrate) of a serum treated with potassium iodide falls to almost one fifth of the original value. Free iodin dissolved with a small amount of potassium iodide also lowers the antitryptic activity. The relation of the iodide to serum antitrypsin will be discussed in a later paper. The influence of iodin in removing the inhibiting action of the serum shows that the inhibiting agents belong to the unsaturated group of the fatty acids.

The effect of hydrogen peroxide in completely destroying the antitryptic activity; the fact that the extracted lipoids, which are the active agents, are all unsaturated compounds, having an iodin value of from fifty to sixty; and also the fact that sera with a high antitryptic index give a higher iodin value than sera with a low antitryptic index, all tend to confirm this view from a chemical standpoint. From the clinical investigations so far reported there is similar confirmation; there is an increase of the antiferment after eating; in the acute infections such as typhoid fever, pneumonia, etc.; in chronic infections such as syphilis and tuberculosis; in Graves' disease; and in the severe anemias, such as those accompanying malignant growths, etc. In all but the first of these conditions, the demands of the body or the action of the toxins, cause a marked inroad into the fat depots of the body. This disappearance of fat in conjunction with a lowering of the oxidation powers of the liver and tissues in general causes an increase in the amount of unsaturated fatty acids in the blood and thus an increase in the ferment-inhibiting action.

CONCLUSIONS.

1. The ferment-inhibiting action of the serum is due to the presence of compounds of the unsaturated fatty acids.

2. These fatty acid compounds may be removed from the serum by means of chloroform or ether.

3. Soaps prepared by saponifying the chloroform or ether extracts inhibit the action of trypsin.

4. The anti-enzyme action of the serum can be removed by filtering acid serum through kaolin, and can in part be restored by extracting the kaolin.

5. The decrease in strength of anti-enzyme in old sera is probably due to the action of the serum lipase.

6. Iodin, potassium iodide, or hydrogen peroxide remove the inhibiting action of the serum.

7. Soaps of the unsaturated fatty acids lose their ferment-inhibiting action when heated with serum at 70° C.

BIBLIOGRAPHY.

1. Brieger, L., and Trebing, J., *Berl. klin. Wchnschr.*, 1908, xlv, 1349.
2. von Bergmann and Meyer, K., *Berl. klin. Wchnschr.*, 1908, xlv, 1673.
3. Meyer, K., *Folia serolog.*, 1911, vii, 472.
4. Eisner, G., *Ztschr. f. Immunitätsforsch., Orig.*, 1908–09, i, 650.
5. Wiens, *München. med. Wchnschr.*, 1907, liv, 2637.
6. Jürgensen, K. F., Inaugural Dissertation, St. Petersburg, 1910; *Centralbl. f. Bakteriol., 1te Abt., Ref.*, 1911, xlix, 29.
7. Halpern, J., *Ztschr. f. Immunitätsforsch., Orig.*, 1911, xi, 630.
8. Weil, R., *Am. Jour. Med. Sc.*, 1910, cxxxix, 714.
9. Morgenroth, J., *Centralbl. f. Bakteriol., 1te Abt.*, 1899, xxvi, 349.
10. Achalme, P., *Ann. de l'Inst. Pasteur*, 1901, xv, 737.
11. Hamburger, W., *Jour. Exper. Med.*, 1911, xiv, 535.
12. Hedin, S. G., *Bio-Chem. Jour.*, 1906, i, 484.
13. Chittenden, R., and Cummins, G., *Jahrb. f. Thierchem.*, 1885, xv, 304.
14. Kudo, T., *Biochem. Ztschr.*, 1909, xv, 473.
15. Bayliss, W. M., *Arch. d. sc. biol.*, 1904, xi, supplement, 261.
16. Abderhalden, E., and Gigon, A., *Ztschr. f. phys. Chem.*, 1907, liii, 251.
17. Schwartz, O., *Wien. klin. Wchnschr.*, 1909, xxii, 1151.
18. Sugimoto, T., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxiv, 14.
19. Cobliner, S., *Biochem. Ztschr.*, 1910, xxv, 494.
20. Kirchheim, L., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxiii, 374.
21. Rosenthal, E., *Folia serolog.*, 1911, vi, 285.
22. Delezenne, C., and Pozerski, E., *Compt. rend. Soc. de biol.*, 1903, lv, 327.
23. Jobling, J. W., and Petersen, W., *Jour. Exper. Med.*, 1914, xix, 239.
24. Jobling and Petersen, p. 383.
25. Thiele, F. H., *Bio-Chem. Jour.*, 1913, vii, 275.

SEROTOXIN.

Studies on Ferment Action. XIV.*

By JAMES W. JOBLING, M.D., and WILLIAM PETERSEN, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

In a previous paper (1) we have demonstrated the lipoidal nature of serum antitrypsin and have discussed the various methods which may be employed to remove the antitrypsin from the serum; *i. e.,* by extraction, or by the saturation or oxidation of the unsaturated carbon bonds of the fatty acids on which the antitryptic property depends. In view of the fact that practically all sera contain proteolytic ferments, we undertook to determine whether or not sera from which the protective substance had been removed, with resulting exposure of the serum proteins, would be toxic for the homologous animal; and if so whether complete removal of the lipoids was necessary to produce toxic effects. Apart from the interest that such a study would have in relation to the production of the so called anaphylatoxins, we felt that numerous pathological conditions, which at present are not wholly understood, might possibly have their basis in a protein intoxication,—a true auto-intoxication in the sense that the toxic substances were formed from the serum or cellular protein of the host, and without reference to the gastro-intestinal tract. We have in mind particularly such conditions as arteriosclerosis, nephritis, asthma, and acute acidosis in infants. The work of Longcope (2) supports this idea. He observed that repeated injections of egg albumen into sensitized dogs and cats caused nephritis and other organic lesions.

Toxicity of Sera which have been rendered Acid and then Filtered.

Our first experiments were made with serum from which the lipoids had been removed by acidifying the serum and then filter-

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ing it through hard paper filters and kaolin, or through a Berkefeld filter. We have shown that part of the antitrypsin can be recovered from such filters by saponification.

0.2 of a cubic centimeter of N/10 sulphuric acid was added to each cubic centimeter of fresh guinea pig serum, and the mixture was filtered several times through a hard paper filter in the evening. It was then placed on ice over night, and on the following morning it was neutralized before injection. In all instances unless otherwise stated the injections were given intravenously.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
4 c.c.	250 gm.	0.016 c.c.

This dose given intravenously caused the immediate symptom complex of anaphylaxis with death of the animal in two minutes. The autopsy findings were typical,—lungs emphysematous, heart beating, and blood fluid.

In the next experiment 4 c.c. of N/4 hydrochloric acid were added to 20 c.c. of guinea pig serum and left over night in the ice box. Before injection it was passed through a Berkefeld filter.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
5 c.c. (not neutralized).	330 gm.	0.015 c.c.

There were marked respiratory convulsions, defecation, scratching of the nose, and the animal was weak for several hours, though it finally recovered.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
5 c.c. (neutralized).	250 gm.	0.02 c.c.

Typical death in three minutes. At autopsy the lungs were emphysematous, the heart was beating, and there were no intravascular clots.

In both of these experiments the serum from which the lipoids had been removed—not merely rendered inactive by acidifying—had become toxic for its own species in doses from 0.016 to 0.02 of a cubic centimeter per gram of weight. The simple acidification of the serum, which inactivates its antitryptic effect, does not seem to make the sera toxic in the same degree, probably because on reinjection the lipoids are again brought into a more active condition. Some toxic symptoms are, however, elicited by such sera in large doses (five cubic centimeters).

TOXICITY OF SERA AFTER EXTRACTION WITH ETHER OR CHLOROFORM.

We have shown that serum antitrypsin can be removed by ether extraction, and that the antitrypsin can be recovered in an almost quantitative relation from the ether extract by saponification of the extracted lipoids. That such sera are toxic is shown in the following experiments.

Guinea pig serum was placed under ether on Jan. 6 at room temperature and was left for two days. The flask was shaken occasionally.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
4 c.c.	330 gm.	0.012 c.c.

Death was immediate. The lungs were emphysematous, the heart had ceased beating, there was a firm clot in the right ventricle, and the gall bladder was distended.

A second preparation of serum was placed under ether on January 8 and permitted to stand at room temperature until January 13. The effect on intravenous injection was as follows:

Dose in c.c.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4 c.c.	330 gm.	0.012 c.c.	Immediate death. Lungs emphysematous. Heart filled with a large clot.
2 c.c.	245 gm.	0.008 c.c.	Immediate death. Findings as above.
1 c.c.	320 gm.	0.003 c.c.	Some respiratory spasms. Looked sick. Complete recovery.

In like manner chloroform will remove the serum antitrypsin, the result being even more rapid than with ether. It is best to mix about two volumes of pure chloroform with one volume of serum and to shake the mixture thoroughly, so that a thick emulsion is formed. After incubation or standing at room temperature the emulsion is centrifuged at high speed, and the supernatant serum is pipetted off and filtered through a coarse filter paper until the serum is quite clear and all the chloroform has evaporated. In the next experiment the serum was extracted with chloroform for two days at room temperature and the serum then removed.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
3.5 c.c.	185 gm.	0.018 c.c.

Death was almost instantaneous. The lungs were emphysematous, the heart beating, and the blood fluid.

A similar preparation was kept at room temperature for five days and then tested.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4.0 c.c.	280 gm.	0.014 c.c.	Immediate death.
2.0 c.c.	310 gm.	0.006 c.c.	Immediate death.
1.0 c.c.	320 gm.	0.003 c.c.	Immediate death.
0.5 c.c.	365 gm.	0.0013 c.c.	Respiratory spasm. Looked sick. Complete recovery.

TOXICITY OF IODIZED SERA.

We have shown that the antitryptic effect of the serum can be completely destroyed by agents that oxidize or saturate the unsaturated bonds of the fatty acids, as for instance with potassium or sodium iodide, free iodin, or hydrogen peroxide. The iodide can not be used for the preparation of serotoxin because to lower the antitryptic index relatively large amounts must be used and these salts in themselves injected intravenously in small doses will produce toxic effects similar to the toxic effects of the serum.

Free iodin dissolved in serum containing only a trace of potassium iodide can be readily used for our purpose, according to the method described by von Dungern and Hirschfeld (3). With an excess of iodin a heavy precipitate is thrown out of the serum, but this is redissolved on the addition of an excess of sodium hydroxide, and the serum can then be brought back to a neutral reaction and will remain clear. It requires about two days at incubator temperature to remove completely the antitryptic action. Such sera remain sterile and will not autolyze if an excess of iodin is added. The results of an intravenous injection of an iodized serum which no longer had any antitryptic effect were as follows:

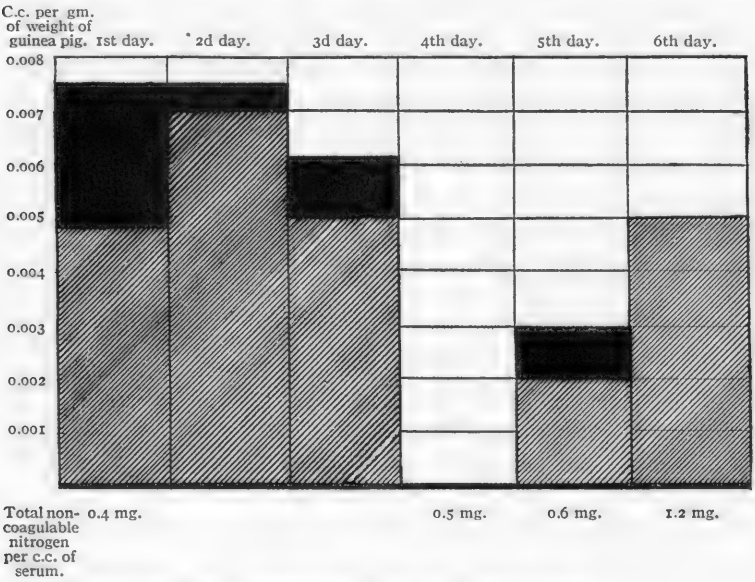
Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
3.2 c.c.	320 gm.	0.01 c.c.	Death in 2 min. Lungs emphysematous. Heart blood fluid. Heart beats strongly.
1.6 c.c.	310 gm.	0.005 c.c.	No effect.

Typical anaphylactic shock was produced with immediate fatal result in a guinea pig which had not been sensitized. This result is of course not comparable to the work of Bruck (4) and Friedberger and Ito (5), who have attempted to show that animals may be sensitized to iodized proteins. It is interesting to note that von Dungern and Hirschfeld (3) observed that their iodized sera underwent autolysis readily, but they could find no explanation for this fact, because they found no difference in the digestion of casein when iodized. They entirely overlooked the fact that the iodin might act on other bodies besides proteins. We have observed no increase in autolysis if an excess of iodin is present because the iodin

itself interferes with ferment action. The serum proteins are, however, digested more easily by trypsin after such treatment. We have been unable to secure toxic sera by treatment with strong hydrogen peroxide.

INFLUENCE OF TIME ON CHLOROFORM EXTRACTION.

We next determined the length of time required for serum kept at room temperature under chloroform to become toxic. For brevity the result has been charted in text-figure 1. The serum before

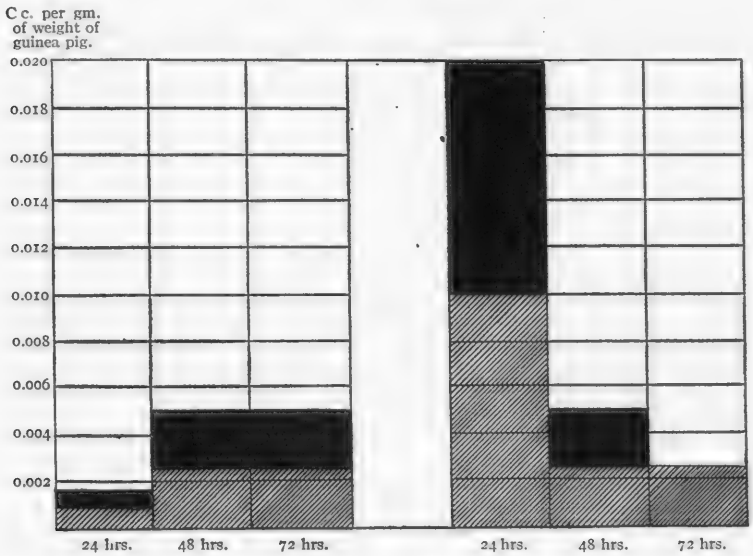


Black = minimum lethal dose per gm. of weight of guinea pig.
Shaded = sublethal dose.

TEXT-FIG. 1. Toxicity of guinea pig serum treated with chloroform at room temperature.

treatment was not toxic in large doses (5 c.c.), and the total non-coagulable nitrogen per cubic centimeter amounted to 0.2 of a milligram. As will be seen from the chart there was a progressive increase in toxicity after the first day until a maximum was reached on the fifth day, when the minimum lethal dose was 0.003 of a cubic centimeter per gram of weight, or 0.75 of a cubic centimeter for a guinea pig weighing 250 grams. There then follows a rather

sudden loss of toxicity, so that 0.005 of a cubic centimeter per gram of weight was no longer toxic. During this time there had been a constant autolysis of the serum, as indicated by the increase in the total non-coagulable nitrogen (Folin's method) from 0.2 of a milligram per cubic centimeter to 1.2 milligrams per cubic centimeter on the sixth day. In each case the minimum lethal dose caused the typical anaphylactic complex, although it was found that excessive



Activated serum under chloroform at 37° C.

Inactivated serum under chloroform at 37° C.

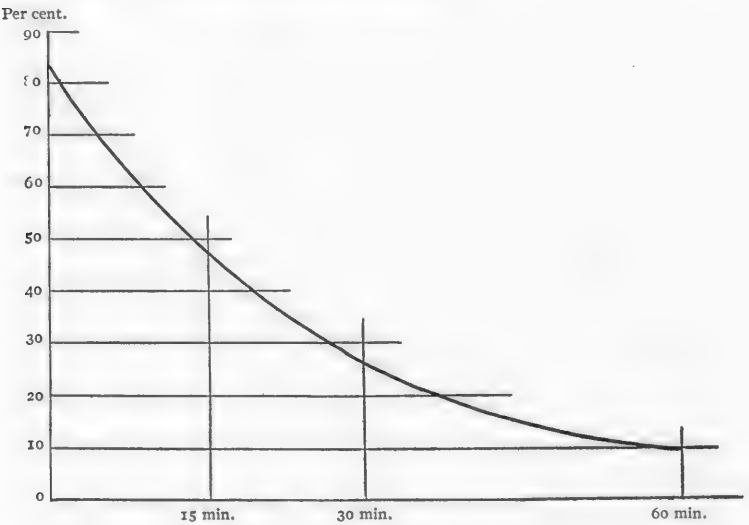
Black = minimum lethal dose in c.c. per gm. of weight.
Shaded = sublethal dose.

TEXT-FIG. 2. Toxicity of guinea pig serum treated with chloroform at 37° C.

doses, two minimum lethal doses, caused thrombosis in the larger vessels and heart.

When left at 37° C. the serum becomes toxic much earlier, and the atoxic stage is correspondingly produced at an earlier period of time (text-figure 2). The active serum so prepared has reached a very toxic stage in twenty-four hours, so that 0.0015 of a cubic centimeter per gram of weight of guinea pig is toxic,—0.31 of a cubic centimeter killed a guinea pig weighing 210 grams,—while the

inactivated serum (fifteen minutes at 54° C.) is toxic only in a much larger dose, 0.02 of a cubic centimeter per gram of weight. After forty-eight hours, however, the active serum has become less toxic, while the inactivated serum has become more toxic than before. Here, as in the previous experiment, there is a constant increase in the amount of non-coagulable nitrogen. In other experiments we have observed that the toxicity will reach its maximum within eight to twelve hours and will then rapidly decline. This depends entirely upon the amount and the rate of removal of the lipoid protective substances. Thus the inactivated serum becomes toxic less rapidly owing to the fact that the antitrypsin is removed more slowly than from the active serum at 37° C.



TEXT-FIG. 3. Curve showing the rate of extraction of serum antitrypsin by chloroform at 37° C.

The rate of removal of the antitrypsin from the serum by chloroform is shown in text-figure 3. In this experiment samples of serum were removed from the incubator at fifteen minute intervals, and the antitrypsin was determined by the method described in our previous papers (6).

0.075 c.c. of normal serum inhibited 84 per cent. of digestion.
After 15 minutes under chloroform it inhibited 48 per cent. of digestion.
After 30 minutes under chloroform it inhibited 27 per cent. of digestion.
After 1 hour under chloroform it inhibited 10 per cent. of digestion.

In one hour practically all the antitrypsin has been extracted. When made strongly alkaline this extraction is markedly accelerated, while the inactivated serum is more resistant to the chloroform extraction during the first half hour, although after about an hour's extraction the lipoids in this case are also removed. The serum is toxic, however, before all the antitrypsin is removed. This is shown by removing a sample after one half hour's incubation. These sera kill in a dose of about 0.01 of a cubic centimeter per gram of weight, and in this case the immediate cause of death is intravascular coagulation. There is no increase in the non-coagulable nitrogen at this time.

PROTOCOL I.

One Hour Chloroform Serum.

Dose.	Weight.	Dose per gm. of weight.	Result.
4.5 c.c.	300 gm.	0.015 c.c.	Immediate death.
3.2 c.c.	270 gm.	0.012 c.c.	Respiratory spasms. Prostrated for 30 min. Recovery.

Two Hour Chloroform Serum.

4.0 c.c.	260 gm.	0.015 c.c.	Immediate death.
1.6 c.c.	215 gm.	0.0075 c.c.	Death in 15 min. Lungs distended. Heart blood fluid. Heart beats.

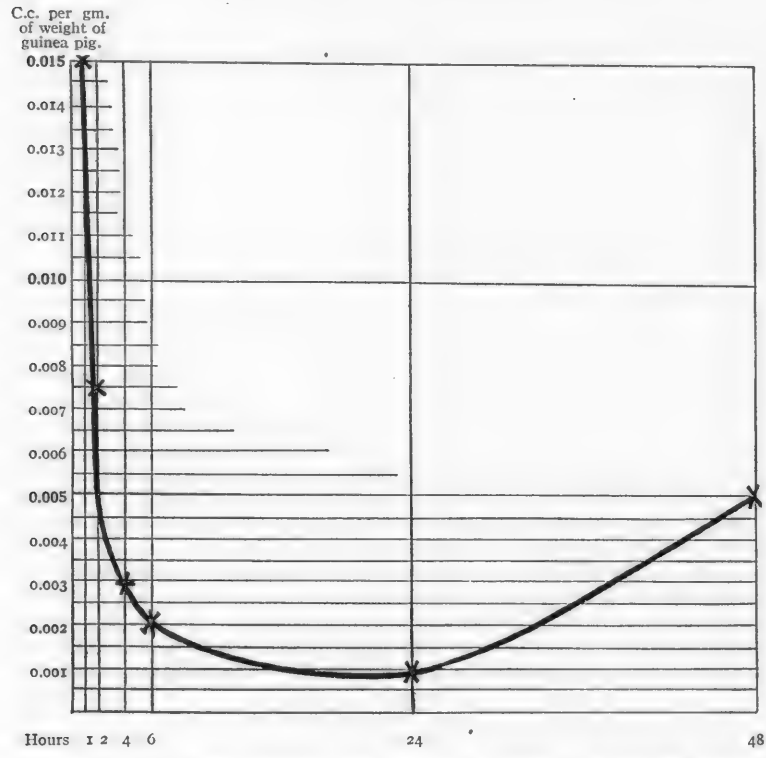
Four Hour Chloroform Serum.

1.7 c.c.	280 gm.	0.006 c.c.	Immediate death. Heart blood clotted. Heart does not beat. Lungs emphysematous.
1.0 c.c.	340 gm.	0.003 c.c.	Death in 30 min. Heart beats. Lungs somewhat distended.

Six Hour Chloroform Serum.

1.1 c.c.	370 gm.	0.003 c.c.	Immediate death. Heart blood clotted. Lungs distended.
0.62 c.c.	310 gm.	0.002 c.c.	Immediate respiratory spasms. Prostrated. Death in 20 min. Small clot in right heart. Blood fluid. Lungs slightly distended.

In text-figure 4 is shown the rate of increase of toxicity observed at 1, 2, 4, 6, 24, and 48 hour intervals of serum kept under chloroform. As will be observed there is a rapid increase during the first six hours, after which the toxicity remains at a constant



TEXT-FIG. 4. Curve showing the increase of toxicity of guinea pig serum treated with chloroform at 37° C. Minimum lethal dose in c.c. per gm. of weight.

level for a period of twelve or more hours, and then rapidly decreases. The minimum lethal doses have been charted from protocol I. The twenty-four and forty-eight hour tests were made at a different time, though with the same lot of serum.

INTRAVASCULAR CLOTTING.

The minimum lethal dose of the toxic sera when given intraperitoneally is much larger, at least five to six times the intravenous dose being required. The immediate cause of death observed in the minimum lethal dose has been either a typical anaphylactic complex, with the lungs emphysematous, the heart beating, and the blood fluid,—observed in the acidified and filtered serum, in the iodized serum, and in the chloroform serum, especially when kept at room temperature; or death has been due to immediate intravascular coagulation with a secondary emphysema, observed in the ether sera, and in the chloroform serum prepared at 37° C. In order to rule out the effect of coagulation we have made the following experiments. The serum used in this experiment had been incubated for two hours at 37° C. The minimum lethal dose was then determined.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Remarks.
1.0 c.c.	340 gm.	0.003 c.c.	Weak. Prostrated. Recovered.
1.1 c.c.	220 gm.	0.005 c.c.	Immediate death. Intravascular coagulation.

Some of this serum was mixed with saline solution to which had been added 1 per cent. sodium citrate, and injected.

Dose.	Citrate.	Weight of guinea pig.	Dose per gm. of weight.
1.25 c.c.	1.25 c.c.	250 gm.	0.005 c.c.

Immediate death. Heart found beating. Lungs emphysematous. Small clot in right heart.

Inasmuch as there was a small clot present we increased the amount of citrate.

Serum.	Citrate.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	2 c.c.	200 gm.	0.005 c.c.

Death in 2 min. Blood fluid. Lungs emphysematous. Heart beating.

Even without a trace of clotting, we find the serum prepared in this way toxic in the same dose, and post-mortem examination shows a typical lung finding.

We next prepared some hirudinized animals and injected them five minutes after treatment with hirudin. In this case each guinea pig received two minimum lethal doses of the toxic sera.

Dose.	Weight of guinea pig.	Amount of serum per gm. of weight.
2.25 c.c.	225 gm.	0.01 c.c.

Typical death in two minutes. No clotting. Lungs emphysematous.

We then used a single minimum lethal dose and also one and one fourth minimum lethal doses, as will be seen below.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
0.825 c.c.	165 gm.	0.005 c.c.
1.0 c.c.	160 gm.	0.0062 c.c.

No immediate result. The animals seemed well and were placed in the cage over night. The next morning both were found dead; in each case the blood was fluid, the lungs emphysematous, although not so markedly as in acute anaphylactic shock.

INHIBITION OF TOXIC ACTION.

Dilution of a single minimum lethal dose with an equal volume of normal guinea pig serum, and incubation for thirty minutes before injection does not alter the toxicity, but heating to 70° C. for thirty minutes greatly decreases it.

If the assumption that we are removing a protective substance from the serum is correct, then by returning the extracted substance to the serum it should become non-toxic. We have previously shown (1) that by saponifying the ether and chloroform extracts of sera we can recover all the antitrypsin. These protective lipoids are not originally present in the serum as soaps, but inasmuch as it is practically impossible to get the lipoids back into solution in the serum in the fine state of dispersion in which form only they are active, we must saponify them, and in the form of soaps attempt to reintroduce them into the serum. Twenty cubic centimeters of guinea pig blood were placed under chloroform for two hours, the serum was then separated and tested for toxicity.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
0.9 c.c.	180 gm.	0.005 c.c.	Immediate death with intravascular clotting, etc.
0.51 c.c.	170 gm.	0.003 c.c.	Scratches; no symptoms.

The minimum lethal dose was therefore 0.005 of a cubic centimeter per gram of weight. The chloroform extract was mixed with an equal volume of alcohol and water and made strongly alkaline with sodium hydrate. This was heated in the water bath for an hour. The mixture was then acidified, and the fatty acids were

taken up in ether and resaponified by neutralization with alcoholic sodium hydrate. These soaps were now mixed with 1.5 cubic centimeters of the serotoxin and incubated for fifteen minutes.

Dose injected.	Weight of guinea pig.	Dose per gm. of weight.
1.1 c.c.	215 gm.	0.005 c.c.

There were no immediate symptoms and the animal remained well. The toxicity had therefore been completely neutralized. In like manner one can completely neutralize the serotoxin by adding soaps of the unsaturated fatty acids, which, as we have shown (6), have marked antitryptic properties, although they are less active when mixed with serum than when dissolved in salt solution. The serotoxin here used was freshly prepared and autolysis had not occurred, so that preformed toxic split products of proteins (primary proteoses) were probably not present. If on the other hand we use a serotoxin which has been prepared at room temperature by standing for several days, in which there is a marked increase in the total non-coagulable nitrogen, and from which one can isolate toxic proteoses, then the neutralization by the soap is no longer complete, the effect depending of course on the relative amount of the proteins and their toxic split products already formed.

A chloroform serum standing for five days at room temperature caused immediate death in doses of 0.003 of a cubic centimeter per gram of weight. Soap was prepared from serum lipoids and mixed with the dose.

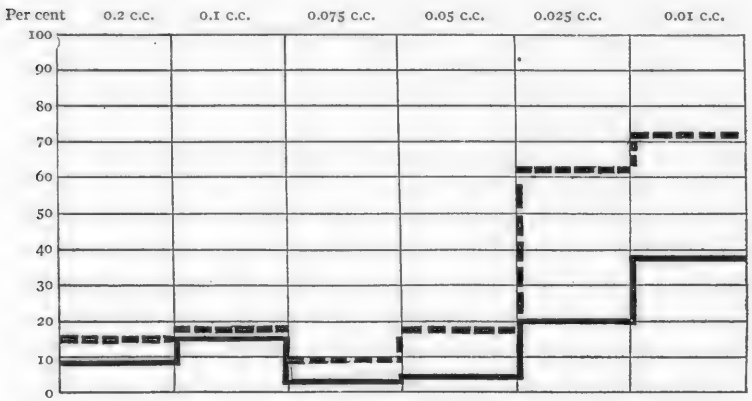
Dose.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	280 gm.	0.0035 c.c.

There were immediate respiratory convulsions, after which the animal recovered for ten minutes; then clonic convulsions occurred at frequent intervals, with death in thirty minutes. In a similar way a dose was prepared by adding 10 mg. of linseed soap (iodin value 110), and in this case, also, while immediate death was prevented, the ultimate result was fatal.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	330 gm.	0.003 c.c.

If the toxicity of the serotoxin depends on the exposure of the proteins, so that they can be easily split to toxic products in the injected animal, then the reduction of the normal antiferments in the injected animal should render the serotoxin more toxic than for a normal animal. A three hour serotoxin (chloroform at

37° C.) was used, the minimum lethal dose of which was 0.005 of a cubic centimeter per gram of weight. Four guinea pigs were prepared in the morning by shaving the abdomen and painting with tincture of iodin, so that each animal received about 0.2 of a gram of iodin. In this way one can reduce the serum antitrypsin in an amount shown in text-figure 5, in which the black line represents the



Black line = before treatment (A. M.).
Dotted line = after being painted with tincture of iodin (P. M.).
TEXT-FIG. 5. Effect of iodin on antitrypsin of guinea pig.

amount of digestion of casein in the presence of varying amounts of the guinea pig serum drawn in the morning before treatment, and the dotted line represents the digestion in the serum of the same guinea pig in the afternoon. This decrease in antitryptic activity is probably due to the saturation by the iodin of the unsaturated carbon bonds of the fatty acids, as we have stated elsewhere (1).

A sublethal dose was first injected into an iodized guinea pig.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.
0.54 c.c.	180 gm.	0.003 c.c.

Immediate death. Heart blood clotted. Lungs slightly distended. Gall bladder markedly distended.

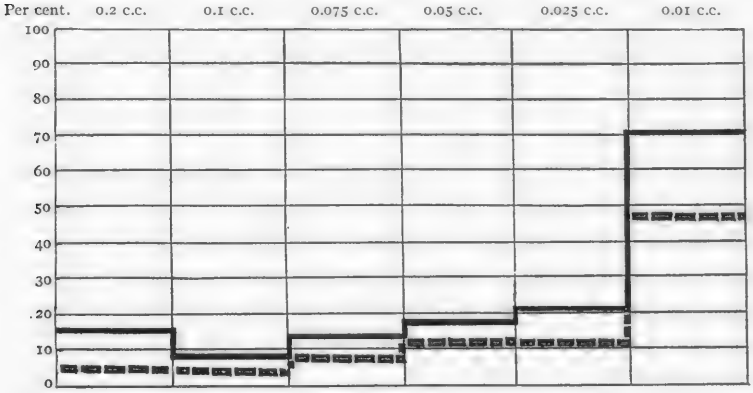
Next a half sublethal dose was given.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.
0.27 c.c.	185 gm.	0.0015 c.c.

The animal was immediately prostrated, sick, with occasional convulsions. Death in thirty minutes. Heart beating. Blood fluid. Lungs slightly distended.

The experiments indicate that the toxicity of the serotoxin is greater for animals in which the antitrypsin has been reduced, thus permitting the ferments normally present to be more active. A single injection of a sublethal dose, given intravenously or intraperitoneally, increases the antitrypsin in the guinea pig; the effect can be demonstrated within twenty-four hours, and persists for at least two weeks. A similar increase has been noted following anaphylaxis (Meyer (7), Pfeiffer (8, 9)). Zinsser (10) has recently noted an increased resistance to a minimum lethal dose of a strong anaphylatoxin prepared from typhoid bacilli, following a single sublethal dose given seven to twelve days previously.

If the serotoxic effects are due to an intravital splitting of the serum protein, then during the period of increased antitrypsin fol-



Black line = original antitrypsin of guinea pig serum drawn on Feb. 24, 1914.
Dotted line = serum drawn on Feb. 28, 1914.

TEXT-FIG. 6. Increase of antitrypsin following a single injection of a sublethal dose of serotoxin.

lowing a sublethal dose there should be an increased resistance to the serotoxin. The effect of a sublethal dose on the guinea pig antitrypsin is shown in text-figure 6. The black line indicates the percentage of casein digestion as influenced by the normal guinea pig serum drawn on February 24, 1914. A sublethal dose of serotoxin was given intraperitoneally on the same day. A second

sample of blood was drawn on February 28, and is shown in the dotted line. As will be seen, there is a well marked increase in inhibiting power.

The increased resistance to serotoxin is shown in the following experiment (February 24).

Dose of serotoxin.	Weight of guinea pig.	Dose per gm. of weight.
1.9 c.c.	190 gm.	0.01 c.c.
Guinea pig sick and prostrated.	Complete recovery.	

The following day the guinea pig received 0.006 of a cubic centimeter, per gram of weight, of a serotoxin preparation of which the minimum lethal dose was 0.005 of a cubic centimeter. There were no symptoms. After several days following the original sublethal dose we have secured animals that are resistant to two minimum lethal doses.

EXPERIMENTS WITH PURIFIED HORSE SERUM ALBUMEN.

In order to compare the results which we had so far obtained with a pure protein intoxication we prepared some horse serum albumen in the following manner: The globulins of the serum were thrown out of solution by half saturation with ammonium sulphate, and after filtration, the albumens were removed from the filtrate by precipitation with acetic acid. This precipitate was redissolved in water made slightly alkaline with sodium carbonate, treated with an equal amount of a saturated solution of ammonium sulphate, filtered, and the filtrate again treated with acetic acid. The final precipitate was dialyzed against running water until free of acid and ammonium sulphate, and was then evaporated to dryness. It was made up in a 1 per cent. solution in normal saline. Of this solution a dose of 0.02 of a cubic centimeter per gram of weight was not toxic for the guinea pig. On testing its antitryptic property it was found that two cubic centimeters of such a solution inhibited 86 per cent. of tryptic digestion, and on digesting two cubic centimeters with a strong trypsin solution for forty-five minutes, only 0.1 of a milligram of non-coagulable nitrogen was obtained. If we now extract the solution by mixing with chloroform at 37° C. for two hours we find the toxicity well marked in the dose which was not at all toxic for the unextracted serum.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4 c.c.	200 gm.	0.02 c.c.	Immediate death. Heart blood clotted. Lungs distended.
2 c.c.	190 gm.	0.01 c.c.	No effect.

After extraction for twenty-four hours the toxicity had still increased.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.	Result.
2.4 c.c.	240 gm.	0.01 c.c.	Killed in 2 min. Heart blood clotted.
0.9 c.c.	180 gm.	0.005 c.c.	No effect.

The last dose, which was sublethal, was now injected into an iodized guinea pig. The method of preparing the guinea pig has been already described.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1.1 c.c.	220 gm.	0.005 c.c.

Immediate death. Heart blood fluid. Lungs distended. Gall bladder distended.

As with the serotoxin, there was a lessened resistance to the toxic effect in the iodized guinea pig. When the antitryptic effect of the extracted (twenty-four hour) horse serum solution was tested, it was found that it now inhibited only 33 per cent. of casein digestion, and was itself digested by trypsin much more easily (0.35 of a milligram for 2 cubic centimeters of solution). After extraction for seventy-two hours the toxicity had increased so that a dose of 0.005 of a cubic centimeter per gram of weight caused marked toxic symptoms but did not kill. At this time the serum albumen was no longer antitryptic. Inasmuch as there are no ferments present in the serum albumen solution, and no trace of autolysis can be obtained when such solutions are incubated, there is no preformation of toxic split products, and whatever increase in toxicity occurs must be due to the increased availability of the serum albumen for attack by the ferments of the injected animal.

In the serotoxin we have a somewhat different condition due to the presence of quite active proteolytic ferments. It would therefore seem reasonable that the ferments would attack the serum proteins as soon as the antitrypsin had been completely removed, and

produce toxic split products,—primary proteoses. This condition actually obtains. If one fractionates a serotoxin which has been under chloroform at 37° C. for only two hours, only faint traces, or no evidence at all, of primary proteoses can be obtained. From twenty-four hour serotoxin we have obtained toxic proteoses, as is shown in the following experiment.

Ten cubic centimeters of normal guinea pig serum were mixed with chloroform and incubated for twenty-four hours. The chloroform was then removed, the native protein removed from the serum by the heat and acid method, and the filtrate made faintly alkaline to throw out the acid albuminates, and again filtered through kaolin. The primary proteoses were then precipitated by half saturation with ammonium sulphate. These were removed, dialyzed until free from all traces of ammonium sulphate, and then made up to the original volumes. When injected intravenously in 0.1 per cent. salt solution the primary proteoses are toxic in a dose of about 0.01 cubic centimeter per gram of weight. When so prepared the toxicity is unaltered by boiling.

DISCUSSION.

In the preliminary experiments we observed the marked similarity to anaphylactic death which our animals showed on the injection of the serotoxin, and later, while working with the rapidly prepared chloroform sera, the paradoxical similarity to cytotoxic death—immediate coagulation—was noted. In view of the fact that the usual method of death is intravascular coagulation, we have used the term serotoxin to designate the entire toxic effect of the extracted sera, although we wish to emphasize the importance of the protein cleavage phenomena.

The toxicity of organ extracts first noted by Brieger and Uhlenhuth (11), was shown by Blaizot (12), Gley (13), and by Dold and Ogata (14) to depend on the intravascular coagulation, and could be rendered completely inert by the treatment of animals with hirudin. Ichikawa (15) has recently shown that sodium citrate will also render a toxic dose non-toxic. Serotoxin differs fundamentally from the cytotoxin in that the toxic effects persist when coagulation is prevented by hirudin or citrate. The toxicity is due to

factors quite apart from the altered coagulation balance, although we believe that it will be found that the unsaturated lipoids play an important part in the mechanism of coagulation and its prevention.

From our experiments we believe that we are justified in concluding that the exposed homologous serum proteins are capable of being split when injected into an animal, and produce the observed toxic effect. In many of our experiments the exact picture of acute anaphylactic death was observed; in sublethal doses a marked fall in temperature and extreme prostration were noted, the latter being most marked in sublethal doses of sera which had been permitted to autolyze. The toxicity of serum protein is well illustrated in the experiment with purified horse serum albumen which was not toxic in doses of 0.02 of a cubic centimeter of a 1 per cent. solution per gram of weight, but when extracted with chloroform it gradually became more and more toxic until a final dose of 0.005 of a cubic centimeter per gram of weight (or 0.00005 of a gram of serum albumen per gram of weight) was sufficient to cause marked toxic effects. The length of time required to extract the lipoids from this solution indicates how intimate the relation of the lipoid to the serum albumen complex must be.

That the exposed serum proteins are the substances which produce *in vivo* the toxic effects when split, is shown by the fact that in the rapidly prepared serotoxin no increase in non-coagulable nitrogen is demonstrable, and that no toxic split products (primary proteoses) can be isolated by fractioning the serum proteins at this time. The antiferments having once been extracted, there is of course no further impediment to ferment action, such sera become actively proteolytic, as was first shown by Delezenne and Pozerski (16), and undergo autolysis as shown by the marked increase in the non-coagulable nitrogen. Under these conditions we have always been able to isolate preformed toxic split products. The effect of the soaps, too, in not completely neutralizing the serotoxin at this period is confirmatory evidence, since the soaps act by preventing the splitting of native proteins (true antitryptic effect, rather than anti-ereptic). With continual autolysis we should expect lessened toxicity, and this is found to be the case. That the

normal inhibitory mechanism of the serum markedly influences the intravital splitting, as it does in the test-tube, is shown by the great increase of toxicity of the serotoxin for animals with lessened antiferments and by the greater resistance of animals with increased antiferments. We believe that the effect of the iodin is due to a saturation of the unsaturated bonds of the fatty acids of the lipoids, rendering the substituted product less active as an antitryptic agent. The effect of a single sublethal dose of serotoxin in increasing the antiferment can possibly be explained by a lowered oxidation of the organism due to the toxin, with a resulting lowering of the physiological oxidation of these unsaturated compounds, or it may be due to a breaking up by the toxic action, of protein-lipoid combinations with a resulting increase in the lipoidal elements in the blood. From our studies we are even more firmly convinced of the biological importance of the protein-lipoid combination in the organism, and the correlated necessity of according to lipolytic action greater emphasis in the part of the adjunct which breaks up these combinations before actual proteolytic changes can take place. The small amount of purified horse serum albumen (after complete chloroform extraction) required to kill a non-sensitized animal is significant from this point of view.

A large number of investigators, notably Vaughan (17), Friedberger (18), and Pfeiffer (9), have, during the past few years, emphasized the importance of the split products of proteins in the production of the toxic effects in the various infections, but the basic idea has always been held that the substrate which was acted upon was the foreign protein concerned, and that the homologous proteins might be neglected, although the work of Keysser and Wassermann (19), Doerr (20), Bordet (21), and Ritz and Sachs (22) indicate that adsorption phenomena and colloidal changes might render homologous sera toxic. The idea, however, that the adsorbed substances were the antiferments, or protective agents, has not been expressed, and no efforts have been made to remove such antiferments, possibly because of the unfortunate view that the antiferments represented immune bodies or split products of the protein molecule.

From a clinical point of view we are inclined to believe that the

proof of the toxicity of exposed serum proteins may be of importance. Longcope (2) has recently shown that repeated injections of heterologous proteins can cause marked nephritic changes. The profound prostration shown by leukemics in the terminal stages when the serum ferments are known to increase greatly, so that such sera may become proteolytic, suggests the profound prostration observed in experimental animals injected with sublethal doses of autolyzed sera. Further experiments are now in progress to determine the results from chronic sero-intoxication.

CONCLUSIONS.

1. Sera from which the protective lipoids (unsaturated fatty acids) have been removed are toxic for the homologous animal.

2. The toxicity is due to three factors: (a) an alteration in the mechanism of coagulation, with resulting intravascular coagulation; (b) the exposure of the native serum proteins; (c) the formation of toxic split products (primary proteoses) by autolysis.

3. A definite maximum of toxicity can be determined, with a final stage of atoxicity due to continued autolysis.

4. Hirudin and sodium citrate do not protect animals.

5. Heating to 70° C. destroys, or greatly lessens, the toxicity of the serotoxin, although the isolated proteoses are toxic after boiling.

6. The return of the extracted lipoids (saponified) neutralizes the toxicity.

7. Unsaturated soaps also neutralize the toxicity.

8. Sublethal doses produce extreme prostration, marked fall in body temperature, no eosinophilia, and an increase of antitrypsin.

9. Sublethal doses of rapidly prepared chloroform sera cause a decrease in coagulation time; sublethal doses of autolyzed sera cause an increase in coagulation time.

10. Previously injected animals are more resistant (increased antiferments).

11. Iodized animals are less resistant (decreased antiferments).

BIBLIOGRAPHY.

1. Jobling, J. W., and Petersen, W., *Jour. Exper. Med.*, 1914, xix, 450.
2. Longcope, W. T., *Proc. N. Y. Path. Soc.*, 1914, xiii, 120.

3. von Dungern, E., and Hirschfeld, L., *Ztschr. f. Immunitätsforsch., Orig.*, 1911, xi, 557.
4. Bruck, C., *Berl. klin. Wchnschr.*, 1910, xlvii, 517, 1928.
5. Friedberger, E., and Ito, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1911–12, xii, 241.
6. Jobling and Petersen, *Jour. Exper. Med.*, 1914, xix, 239.
7. Meyer, K., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xix, 179.
8. Pfeiffer, H., and Jahrisch, A., *Ztschr. f. Immunitätsforsch., Orig.*, 1912–13, xvi, 38.
9. Pfeiffer, H., and de Crinis, M., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xvii, 459.
10. Zinsser, H., Personal communication.
11. Brieger and Uhlenhuth, *Deutsch. med. Wchnschr.*, 1898, xxiv, 163.
12. Blaizot, L., *Compt. rend. Soc. de biol.*, 1911, lxxi, 534.
13. Gley, E., *Compt. rend. Soc. de biol.*, 1912, lxxii, 7.
14. Dold, H., and Ogata, S., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xiii, 667.
15. Ichikawa, S., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xviii, 163.
16. Delezenne, C., and Pozerski, E., *Compt. rend. Soc. de biol.*, 1903, lv, 327, 690, 693.
17. Vaughan, V. C., *Jour. Am. Med. Assn.*, 1914, lxii, 583.
18. Friedberger, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 369.
19. Keysser, F., and Wassermann, M., *Ztschr. f. Infectionskrankh.*, 1911, lxviii, 535.
20. Doerr, R., *Wien. klin. Wchnschr.*, 1912, xxv, 331.
21. Bordet, J., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 877.
22. Ritz, H., and Sachs, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 987.

FURTHER OBSERVATIONS ON THE GROWTH OF BACTERIA ON MEDIA CONTAINING VARIOUS ANILIN DYES, WITH SPECIAL REFERENCE TO AN ENRICHMENT METHOD FOR TYPHOID AND PARATYPHOID BACILLI.*

By CHARLES KRUMWIEDE, Jr., and JOSEPHINE S. PRATT.

(*From the Research Laboratory of the Department of Health, New York.*)

In a previous communication (1) we reported the results of a series of observations on the growth of thirty varieties of bacteria on media containing various dyes. We have continued this work using the same series of cultures and forty samples of dyes. The methods employed have been the same. Green dyes are widely used in special media for typhoid and paratyphoid isolation. This led us to include many of these dyes in our present work, in the hope that some might be more selective than those in use. If this were the case, their application to plate and fluid media would be of great value.

In general, the results have been similar to those with other dyes, especially gentian violet and its allies (table I). The inhibition of growth has been most evident among the Gram-positive bacteria, the Gram-negative bacteria as a rule growing freely on the dyed agar.

Certain exceptions are evident in the table. The cholera strain employed was less resistant to the action of green dyes than the allied vibrios. To determine whether this was of differential value, forty cultures of cholera and other vibrios were tried on agar containing *Bittermandelölgrün,* the dye showing the greatest differences. A variable restraining action was noted, but there were no differences according to types.

The diphtheria bacillus and its allies have given apparent differences and irregularities. This is due to the tendency of these cultures to produce flakes which, when inoculated on the agar, produce a limited growth, whereas the separate bacilli fail to grow.

* Received for publication, February 18, 1914.

TABLE I.[1, 2]

	Bacteria tested.	Brilliant green, crystals 3.66 (Bayer).			Brilliant green.			Anilin green.			Solid green.			Victoria green, crystals 4.813 (Bayer).			Smaragd green.			China green.			Malachite green crystals, "Chlorzink-doppelsalz."			Bitter-mandelölgrün.			Victoria blue 4 R.			Malachite green.			Malachite green (Höchst).		
		1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000
Gram-positive	Staphylococcus aureus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*	–	–	×	–	–	*	–	–	*	–	–	–	–	–	×	–	–	×	–	–
	Streptococcus (green)	–	–	–	×	–	–	–	–	–	–	–	–	–	–	–	–	–	–	∓	–	–	*	–	–	∓	–	–	∓	–	–	×	–	–	×	–	–
	Streptococcus (hemolytic)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	×	–	–	–	–	–	×	–	–	×	–	–	–	–	–	–	–	–
	Streptococcus mucosus capsulatus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	×	–	–	×	–	–	*	–	–	–	–	–
	Pneumococcus	–	–	–	–	–	–	–	–	–	–	–	–	∓	–	–	–	–	–	×	–	–	–	–	–	*	–	–	*	–	–	–	–	–	–	–	–
	B. diphtheriæ	×	–	–	×	–	–	–	–	–	–	–	–	–	–	–	–	–	–	×	–	–	–	–	–	×	–	–	×	–	–	×	–	–	×	×	–
	Diphtheroid bacilli (Cameron)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	×	–	–	×	–	–	×	–	–	×	–	–
	Diphtheroid bacilli (Cunningham)	×	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	∓	–	–	∓	–	–	×	–	–	×	–	–
	B. hoffmanni	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	×	–	–	∓	–	–	∓	–	–	×	*	–	×	–	–
	B. subtilis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	×	–	–	×	–	–
	B. lactimorbi	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*	–	–
	B. anthracis	–	–	–	–	–	–	–	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*	–	–	∓	–	–
Gram-negative	Cholera	*	–	–	*	–	–	–	–	–	–	–	–	∓	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+	×	*	+	+	×
	Vibrio (indol-positive)	*	–	–	×	*	–	×	–	–	–	–	–	∓	×	*	×	×	–	+	×	–	+	∓	×	+	∓	×	+	+	∓	+	+	∓	+	+	+
	Vibrio (indol-negative)	∓	–	–	+	×	–	+	–	–	∓	×	–	∓	*	*	∓	×	*	+	∓	*	+	×	*	+	∓	–	+	∓	–	+	∓	+	+	+	+
	B. proteus	∓	*	*	–	×	*	+	*	*	∓	×	–	+	∓	×	+	×	*	+	∓	×	+	∓	∓	+	∓	×	+	∓	×	+	+	+	+	+	+
	B. pyocyaneus	+	+	+	+	+	+	+	+	+	+	+	+	+	∓	×	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. dysenteriæ (Shiga)	×	–	–	∓	×	–	∓	–	–	+	*	–	+	–	–	+	*	–	+	*	–	–	+	–	–	+	–	–	+	+	+	∓	*	+	+	+
	B. dysenteriæ (Flexner)	–	–	–	×	×	–	–	–	–	–	–	–	+	–	–	×	–	–	+	*	–	–	+	–	–	+	–	–	+	+	+	+	∓	*	+	∓	×
	B. dysenteriæ (Mt. Desert)	×	–	–	×	×	–	×	–	–	×	–	–	+	–	–	×	–	–	+	*	–	–	+	–	–	+	–	–	+	+	+	+	∓	*	+	∓	∓
	B. typhosus	+	×	–	+	×	×	+	×	–	+	×	–	+	+	×	+	×	×	+	+	×	+	∓	∓	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. paratyphosus, A	+	+	∓	+	+	∓	+	+	∓	+	+	∓	+	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. paratyphosus, B	+	+	∓	+	+	∓	+	+	∓	+	+	∓	+	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. enteritidis (Gaertner)	+	+	+	+	+	∓	+	+	∓	+	+	∓	+	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Gram-negative Bacteria tested.	Brilliant green, crystals 366 (Bayer).			Brilliant green.			Anilin green.			Solid green.			Victoria green, crystals 4.833 (Bayer).			Smaragd green.			China green.			Malachite green crystals, "Chlorzink-doppelsalz."			Bittermandelölgrün.			Victoria blue 4 R.			Malachite green.			Malachite green (Höchst).		
	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000
B. coli communis	∓	*	−	+	×	−	+	−	−	+	*	−	+	∓	−	∓	×	−	+	+	×	+	∓	∓	+	+	∓	+	+	∓	+	+	+	+	+	+
B. coli communior	∓	−	−	+	×	−	+	−	−	+	×	−	+	∓	−	∓	×	−	+	+	×	+	∓	∓	+	+	×	+	+	∓	+	+	+	+	+	+
Friedländer's bacillus	∓	*	*	+	∓	×	+	×	×	+	∓	×	+	∓	×	+	+	×	+	+	×	+	+	+	+	+	×	+	+	∓	+	+	+	+	+	+
B. lactis aerogenes	×	−	−	+	×	*	+	−	−	∓	×	−	+	∓	*	∓	∓	×	+	+	×	+	+	+	+	+	*	+	+	∓	+	+	+	+	+	+
B. ozenæ	−	−	−	×	−	−	×	−	−	×	−	−	+	∓	−	∓	*	−	+	∓	×	+	+	+	+	+	+	+	+	∓	+	+	+	+	+	+
B. pullorum	+	∓	∓	+	∓	−	+	+	∓	+	+	∓	+	+	∓	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[1] In the tables + = growth like control; ∓ = restrained growth; × = markedly restrained growth; − = no growth; * = some growth developed later; § = several colonies.

[2] In addition to the dyes given in the table, the following were also tested: alkali green, *Säurealizaringrün*, *Lichtgrün* F. S., *Brillantblau* (*extra grünlich*), naphtol green B., *Echtgrün*, *Säuregrün*, alizarin green B., methyl blue, trypan blue, anilin blue (water-soluble), China blue, anilin blue-black, *Blauschwarz*, and *Wasserblau*. In all the dilutions employed growth was as vigorous as on the control. Where not otherwise stated, all the dyes employed were obtained from Grübler.

TABLE I.—*Concluded.*

	Bacteria tested.	Malachite green Ia.			Nachtblau.			Methylene blue, med. pure.			Janus green.			Magenta red.			Methyl green, oo rein.			Chlorhydrin blue, or acetin blue.			Pyronin.			Iodin green.			Alkali blue.			Methylene green.			Methyl green.		
		1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000	1:500,000	1:100,000	1:50,000
Gram-positive	Staphylococcus aureus	×	−	−	∓	−	−	∓	×	*	+	*	−	+	∓	×	∓	∓	*	+	+	+	+	+	+	+	∓	×	+	+	+	+	+	+	+	+	×
	Streptococcus (green)	∓	−	−	∓	−	−	+	−	−	+	×	*	+	∓	×	+	+	+	+	+	+	+	×	−	+	+	+	+	+	+	+	+	+	−	+	+
	Streptococcus (hemolytic)	−	−	−	∓	−	−	+	*	−	+	×	−	∓	×	−	+	+	+	+	+	+	+	*	−	+	+	+	+	∓	+	∓	+	*	+	+	∓
	Streptococcus mucosus capsulatus	*	−	−	×	−	−	+	−	−	+	×	−	+	∓	×	+	+	+	+	+	+	+	×	−	+	+	+	+	+	∓	+	+	∓	+	+	+
	Pneumococcus	−	−	−	∓	−	−	+	−	−	+	∓	−	+	∓	∓	+	+	+	+	+	+	+	∓	−	+	+	+	+	+	∓	+	+	×	+	+	∓
	B. diphtheriæ	∓	×	−	∓	−	−	×	*	−	+	−	−	×	−	−	∓	×	−	×	×	−	+	+	∓	+	∓	×	+	∓	×	+	+	∓	+	+	∓
	Diphtheroid bacilli (Cameron)	×	−	−	−	−	−	−	−	−	+	−	−	+	−	−	×	−	−	×	−	−	+	+	∓	+	×	×	+	×	×	+	+	∓	+	+	∓
	Diphtheroid bacilli (Cunningham)	×	−	−	−	−	−	−	−	−	−	−	−	×	−	−	∓	*	−	×	−	−	+	+	+	+	∓	×	+	×	×	+	+	∓	+	∓	×
	B. hoffmanni	×	−	−	−	−	−	−	−	−	−	−	−	×	−	−	+	*	−	×	−	−	+	+	+	+	∓	×	∓	×	×	+	+	*	+	∓	×
	B. subtilis	×	−	−	∓	−	−	∓	∓	×	+	−	−	+	×	−	+	×	−	+	+	+	+	+	+	+	∓	*	+	+	+	+	+	+	+	+	×
	B. lactimorbi	*	−	−	×	−	−	∓	*	−	∓	−	−	+	×	−	+	∓	*	+	+	+	+	+	+	+	∓	∓	+	+	+	+	+	×	+	+	×
	B. anthracis	×	−	−	∓	−	−	+	+	∓	+	−	−	+	∓	×	+	∓	−	+	+	+	+	+	+	+	∓	×	+	+	+	+	+	+	+	+	∓
Gram-negative	Cholera	+	+	×	+	+	+	+	×	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	×	+	+	+
	Vibrio (indol-positive)	+	+	+	+	+	+	+	×	×	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Vibrio (indol-negative)	+	+	+	+	+	+	∓	∓	×	+	+	+	+	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. proteus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. pyocyaneus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. dysenteriæ (Shiga)	+	+	+	+	+	+	∓	∓	+	+	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. dysenteriæ (Flexner)	+	∓	×	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. dysenteriæ (Mt. Desert)	+	∓	×	+	+	+	+	∓	+	∓	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. typhosus	+	+	+	+	+	+	+	+	+	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. paratyphosus, A	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. paratyphosus, B	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. enteritidis (Gaertner)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. coli communis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. coli communior	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Friedländer's bacillus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. lactis aerogenes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. ozænæ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. pullorum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

With one exception the results obtained have failed to show variations which could be used for practical differentiation. They are given, however, as similar detailed observations are not available in the literature.

The observations on the green dyes show marked variations in the resistance of members of the typhoid-paratyphoid-colon group. In general the paratyphoid-enteritidis types are highly resistant, the typhoid less so, but somewhat more resistant than the coli types. Various green dyes (2) have been employed in media to suppress the colon types found in feces. Loeffler employed malachite green agar, Werbitzki China green, and Conradi brilliant green agar, adding picric acid which reduces the activity of the dye. The media have been of some use, especially for preliminary plating for enrichment, for paratyphoid more than for typhoid. On these media the difference in resistance of the typhoid and colon types was slight. If very few typhoid bacilli were present or if less resistant than the average, failure resulted. Peabody and Pratt and others attempted to use malachite green in fluid media as an enrichment medium.

Of the dyes we tried, ten revealed variations among the typhoid-colon group. A large number of cultures of the group were obtained[3] and grouped according to their sugar fermentations, and tried on agar containing some of these dyes. This was done to determine whether one dye showed wider differences than another and as a preliminary to the application of the results already obtained, to a selective plating medium, or a fluid enrichment medium. Agar is an easier medium to employ as an index, and was therefore used, as we had found that the results on agar could be duplicated in broth with slight variations in dilutions. To reveal slight differences in reaction, a small number of bacteria were inoculated. A loopful of a broth culture was carried to a second tube and loop inoculations were made from this (table II).

The results show that the different dyes possess a similar differential action. This difference, however, appears at different dilutions according to the dye employed. At the appropriate dilutions no one dye is perceptibly more differential than another.

[3] We are indebted to Dr. Torrey and Prof. C.-E. A. Winslow for some of the cultures employed.

TABLE II.[4, 5]

Type tested.	No. of strains.	Glucose.	Dulcite.	Lactose.	Raffinose.	Saccharose.	Anilin green. 1:300,000	Anilin green. 1:100,000	Anilin green. 1:50,000	Brilliant green (Grübler). 1:300,000	Brilliant green (Grübler). 1:100,000	Brilliant green (Grübler). 1:50,000	Brilliant green (Bayer). 1:300,000	Brilliant green (Bayer). 1:100,000	Brilliant green (Bayer). 1:50,000	Smaragd green. 1:100,000	Smaragd green. 1:50,000	Smaragd green. 1:10,000	Solid green. 1:100,000	Solid green. 1:50,000	Solid green. 1:10,000	Victoria green (Bayer). 1:100,000	Victoria green (Bayer). 1:50,000	Victoria green (Bayer). 1:10,000	China green. 1:100,000	China green. 1:50,000	China green. 1:10,000
B. alkaligenes	2	−	−	−	−	−	−	−	−	−	−	−	..	..	..	−	−	−	−	−	−	..	..	..	..	..	..
B. typhosus	3	+	−	−	−	−	ǂ	*	−	×	*	−	ǂ	*	−	−	−	−	×	−	−	ǂ	×	*	ǂ	×	×
B. paratyphosus, A	6	†	†	−	−	−	+	ǂ	×	+	+	×	+	ǂ	×	ǂ	ǂ	×	+	+	×	+	+	+	+	+	+
							ǂ	*	−	ǂ	×	−	ǂ	×	*	−	−	−	§	−	−	ǂ	ǂ	−	ǂ	ǂ	ǂ
B. paratyphosus, B	4	†	†	−	−	−	+	+	ǂ	+	+	ǂ	+	+	ǂ	ǂ	ǂ	ǂ	+	ǂ	ǂ	+	+	+	+	+	+
							+	ǂ	−	+	ǂ	−	ǂ	ǂ	×	×	*	−	ǂ	×	*	+	+	ǂ	+	+	ǂ
B. enteritidis	2	†	†	−	−	−	+	+	×	+	ǂ	×	+	ǂ	ǂ	+	ǂ	×	ǂ	ǂ	×	+	+	+	+	+	ǂ
							+	ǂ	−	+	ǂ	−	ǂ	×	−	×	*	−	ǂ	ǂ	−	+	+	+	+	+	+
Intermediate types	4	†	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	ǂ	−	−	ǂ	ǂ	×
	1	†	−	−	+	−	−	−	−	−	−	−	..	..	..	−	−	−	−	−	−	..	..	..	..	..	..
	1	†	†	+	†	†	−	−	−	*	−	−	−	−	−	−	−	−	−	−	−	ǂ	×	§	ǂ	ǂ	×
	1	†	†	+	†	+	−	−	−	−	−	−	..	..	..	−	−	−	−	−	−	..	..	..	..	..	..
B. acidi lactici	6	†	−	†	−	−	−	−	−	−	−	−	..	..	..	−	−	−	−	−	−	..	..	..	..	..	..
B. coli communis	3	†	†	†	† or −	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. coli communior	6	†	†	†	†	†	+	+	×	+	ǂ	ǂ	+	ǂ	ǂ	ǂ	×	×	ǂ	ǂ	×	+	+	+	+	ǂ	+
							−	−	−	−	−	−	×	−	−	−	−	−	−	−	−	×	*	−	ǂ	×	*
B. aerogenes types	6	†	−	†	†	†	+	+	ǂ	+	ǂ	ǂ	+	+	ǂ	ǂ	ǂ	ǂ	+	ǂ	ǂ	+	+	+	+	+	+
							−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[4] Where two sets of observations are given they are the extremes on that type of culture.

[5] − = no fermentation; + = acid production; † = production of acid and gas; .. = no observations. Other symbols are as in preceding table.

Five cultures were not included in the preceding table. These are the cultures used by Churchman and Michael (3). We have taken their observations on the growth of these cultures on gentian violet agar and their interagglutinations, and added our observations of the sugar fermentations of the same cultures and of the growth on media containing green dyes. Table III shows the possible application of dye media to the differentiation of some members of the enteritidis group. The question that immediately arises is: Has the reaction to dyes any relation to their source or pathogenicity? For instance, two cultures, E 25 and E 26, were isolated from a drainage canal by Jordan. Unfortunately the source of the other cultures is not available. If the strains which did not grow came from human beings suffering from infection by these organisms, there will be times when the application of these dyes for enrichment methods will fail.

TABLE III.

Organism.	Growth on gentian violet agar.	Growth on agar, or in broth with green dyes.[6]	Glucose.	Dulcite.	Summary of agglutination reactions. Sera.			
					E 18.	E 25.	E 26.	E J.H.H., same as E 234.
E 18 (67).....	+	+	†	†	+++	0	0	++
E 25 (68).....	+	−	†	−	0	+++	0	0
E 26 (14).....	+	−[7]	†	†	0	0	+++	0
E 132 (70).....	+	+	†	†	+++	0	0	++
E 234 (64).....	−	−[7]	†	†	++	0	0	+++

Experiments were then begun with fluid and solid media for differential enrichment. The use of solid media was not as successful as fluid media. As we had not found any one dye more selective than another and because of two reports giving results similar to our own which appeared at this time, we continued to work with brilliant green.

Torrey (4) found that by the use of Grübler's brilliant green in fluid media he could enrich the paratyphoid-enteritidis group and restrain the common fecal bacteria. Browning, Gilmour, and Mackie (5) were able, by the use of Bayer's brilliant green in fluid

[6] Dyes given in table II.

[7] Very slight delayed growth on agar containing 1 : 500,000 of some of the dyes.

media, to isolate typhoid or paratyphoid bacilli from stools, where direct plating was unsuccessful.

Preliminary tests were made to determine what medium to use. Peptone and meat extract broth with and without glucose and containing the dye in various dilutions were tried. Counts were made of the number of paratyphoid bacilli inoculated and the number per loop of the broth after incubation. The glucose extract broth gave the most abundant growth. This medium was therefore used in our subsequent work. For all our work we have used media neutral to phenolphthalein.

Some experiments had shown that slight changes would lower the threshold of growth for the typhoid bacillus but make little change in the restraining action of the dye towards the coli types; for instance, small additions of proteid substances change the action of the dye. A test was made, therefore, of the influence of added feces as a preliminary to selecting dilutions for work with stools. Typhoid bacilli, being less resistant, were used for this test. The results with Bayer's brilliant green (table IV) show that about one third of the activity of the dye is lost.

TABLE IV.

	No. per loop after 18 hours' incubation.		
	500,000.	400,000.	300,000.
Typhoid (213 bacilli inoculated)	218	0	0
Typhoid +0.1 c.c. of boiled feces (213 typhoid bacilli inoculated)	+++[8]	+++	+++
0.1 c.c. of boiled feces (control)	0	0	0

As a further index to the selection of dilutions, the possible variation of different strains to the dye and to different types of the dye was determined. Further strains were tried in broth containing Bayer's brilliant green. The number of bacilli inoculated ranged from one to seventy. No growth, as determined by plating a loopful of the media, occurred in dilutions after and including 1 to 400,000. In 1 to 500,000 two showed multiplication. A loop plated from these tubes showed only 24 and 500 colonies. Repeating this with six strains with Grübler's dye gave essentially similar results.

[8] Like plain broth control.

TABLE V.[9]

Results of Examination of Stools.

Name of case.	Direct plates (Conradi).	Conradi plates after enrichment in broth containing brilliant green.			Source of dye.
		1:500,000	1:400,000	1:300,000	
R.	Less than 1% typhoid	10% typhoid[10]	Over 50% typhoid; many bluish colonies not typhoid	Sterile (lower dilutions also sterile)	Bayer.
M.	Negative (overgrowth by red colonies)	60% typhoid	75% typhoid	Overgrown by aerogenes types (lower dilutions the same)	Bayer.
O.	30% typhoid	Overgrown by aerogenes types	Many hundred typhoid colonies wherever plate is free from aerogenes types	Overgrown by aerogenes types; typhoid isolated from plate (lower dilutions gave aerogenes types only)	Bayer.
M.	7% typhoid	90% typhoid	95% typhoid	95% typhoid	Grübler.
M.	7% typhoid	Many typhoid colonies where plate is clear of heavy red growth; plate red from diffusion	Clear parts of plate peppered with typhoid colonies; heavy red growth with diffusion of acid through plate	Moderate number of typhoid colonies; plate overgrown by aerogenes types	Bayer.
O.	50% typhoid	Very many typhoid colonies where plate is free from overgrowth of aerogenes types	Few typhoid; practically overgrown by aerogenes types	Moderate number of typhoid colonies; plate overgrown by aerogenes types	Grübler.
O.	50% typhoid	Very many typhoid colonies where plate is free from overgrowth of aerogenes types	Few typhoid; practically overgrown by aerogenes types	Plate overgrown by mucosus types	Bayer.
R.	Five typhoid colonies or three plates	Confluent growth, red margin and bluish center, many fine bluish colonies between confluent areas; impossible to identify typhoid by direct agglutination from plate; fishings gave slow lactose-fermenting aerogenes types.			Grübler.
R.	Five typhoid colonies or three plates	Confluent growth, red margin and bluish center, many fine bluish colonies between confluent areas; impossible to identify typhoid by direct agglutination from plate; fishings gave slow lactose-fermenting aerogenes types.			Bayer.

[9] Eight other stools were examined and were negative both by direct plating and after growth in dye broth.

[10] Verified by direct agglutinations of growth from plate or by fishing onto Russell's medium with subsequent agglutination.

Two strains, however, showed slight multiplication at the 1 to 400,000 dilution.

Because of these results dilutions of 1 to 500,000 to 1 to 100,000 were tried in testing the stools from typhoid carriers. The stools were from three cases and were examined on two occasions, once with Bayer's dye only, and once with both makes of dye. The broth was used in ten cubic centimeter amounts, and 0.1 of a cubic centimeter of thinned feces was added to each tube. The results are given in table V.

That we can enrich typhoid by this method is evident. The results confirm those of Browning, Gilmour, and Mackie. We also agree with their suggestion to use graded dilutions. When we add feces we are introducing a variable factor and the typhoid strains also vary somewhat in their resistance to the dye. Success depends on the right adjustment. The value of the method must rest on the results of routine examinations over a period of time. From our results we would suggest the use of the three dilutions as given, and the addition of 0.1 of a cubic centimeter of thinned feces. The overgrowth of some of the plates suggests the advisability of streaking two plates from each tube, without burning the wire between the plates. Unsuccessful results from overgrowth are apparently due most frequently to *Bacillus aerogenes* types, which are extremely resistant to the dye and usurp the plate where seeding is too heavy.

The range of variability of the available paratyphoid-enteritidis strains was determined. Table VI gives the results.

The results show a wide range of variability. Certain members of this group, as already noted, failed completely to grow. How far such variations will be present in strains in human feces will be shown only by the results of routine examinations.

The results with our cultures make us doubt the advisability of using only one dilution,—1 to 6,600, as advised by Torrey. Although he grades the amount of feces, we doubt whether this would reduce the activity of the dye sufficiently to allow the growth of the less resistant types, assuming that such strains will occur in naturally infected feces. Furthermore, such low dilutions may not weed out the more resistant fecal types of bacteria any more than would the

TABLE VI.[11]

Culture and type.		No. inoculated.	Brilliant green (Bayer). No. per loop after 18 hours. 1 : 100,000	Brilliant green (Bayer). No. per loop after 18 hours. 1 : 10,000	No. inoculated.	Brilliant green (Grübler). No. per loop after 18 hours. 1 : 100,000	Brilliant green (Grübler). No. per loop after 18 hours. 1 : 10,000
Paratyphoid, A	3	53	+|	+	92	++	1320
	54	228	++	242	300	+|	99
	56	364	++	++	496	++	++
	57	392	++	+|	248	+|	85
	58	314	++	+|	640	++	++
	60	304	++	++	47	++	++
	62	59	++	0	112	0[12]	0
	63	234	2[12]	0	400	2[12]	0
	66	200	++	++	240	++	++
Paratyphoid, B	52	600	++	+|	412	+|	214
	53	282	++	++	55	++	++
	59	85	++	0	18	0[11]	0
	61	140	++	+|	228	++	++
	65	142	++	+|	160	++	++
Enteritidis	4	384	4[12]	0	668	552[12]	129
	47	270	++	++	160	++	+|
	67	360	++	++	352	++	++
	70	298	++	++	480	++	+|

higher dilutions. Some aerogenes types are more resistant than the most resistant paratyphoid-enteritidis strains. In fact two of Torrey's strains showed decidedly less resistance than the others, and acted more like some of our strains. His results with feces of dogs artificially infected prove the value of the method, but are no index to the correct dilutions to employ. The strains used for feeding are not stated, but were undoubtedly fully resistant to the dye. This proves nothing as to the results to be expected in routine examinations where strains may vary.

As no naturally infected stools were at our disposal, we did not continue work in this direction. From our results with cultures, graded dilutions seem to us to be indicated in stool examinations for paratyphoid and enteritidis. Taking into consideration the fact that the feces itself will lower the activity of the dye, a series of dilutions ranging from 1 to 100,000 to 1 to 500,000 will probably cover the variations we have noted, with the exception of those

[11] ++ = closely crowded colonies, like control; +| = closely crowded colonies, less than control; + = closely crowded colonies, less than preceding.

[12] Quantitatively similar restraint in 1 : 200,000.

strains which were completely inhibited, and restrain the fecal organisms that can be restrained.

Our results in the attempt to apply these results to a solid medium have been only partially successful. The green dyes can be used as by Conradi, but this has the disadvantage of the absence of an indicator for lactose fermentors. The use of sodium sulphite with the green dye or some other added dye, as fuchsin, has the disadvantage that the sulphite lowers the activity of the dye and a balance is obtained with difficully. The attempt to find an indicator which would be sharp but not influence the activity of the green dye has so far been unsuccessful.

CONCLUSIONS.

Several green dyes show a marked selective action for members of the typhoid-paratyphoid-colon group. This can be used for the enrichment of typhoid and paratyphoid bacilli present in feces. Forty dyes were tested with thirty strains covering all types of pathogenic bacteria. In general the dyes restrained the growth of the Gram-positive bacteria but had no effect on the growth of the Gram-negative group.

BIBLIOGRAPHY.

1. Krumwiede, C., Jr., and Pratt, J. S., Observations on the Growth of Bacteria on Media Containing Various Anilin Dyes, *Jour. Exper. Med.*, 1914, xix, 20.
2. Kutscher, K. H., Abdominaltyphus, in Kolle-Wassermann, Handbuch der pathogenen Mikroorganismen, 2d edition, Jena, 1913, iii, 747–757.
3. Churchman, J. W., and Michael, W. H., The Selective Action of Gentian Violet on Closely Related Bacterial Strains, *Jour. Exper. Med.*, 1912, xvi, 822.
4. Torrey, J. C., Brilliant Green Broth as a Specific Enrichment Medium for the Paratyphoid-Enteritidis Group of Bacteria, *Jour. Infect. Dis.*, 1913, xiii, 263.
5. Browning, C. H., Gilmour, W., and Mackie, T. J., The Isolation of Typhoid Bacilli from Feces by Means of Brilliant Green in Fluid Medium, *Jour. Hyg.*, 1913, xiii, 335.

FACTORS OF RESISTANCE TO HETEROPLASTIC TISSUE-GRAFTING.

Studies in Tissue Specificity. III.*

By JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 56 to 60.

Previous observations have tended to show conclusively that tissues cannot be transplanted from one species to another, even though these be closely related. Two theories have been brought forward to explain this failure in heteroplastic grafting. The two schools are still at variance and neither has been able to produce evidence conclusive enough to convince the other. The first and most prominent theory is that of Ehrlich, termed athrepsia.[1] The experimental foundation for this hypothesis is the so called zigzag transplantation of tumors between rats and mice. It was observed that a mouse tumor when grafted into a rat, or *vice versa,* would survive and proliferate for six to eight days, but would later fail rapidly and be absorbed. If, however, the mouse tumor was removed during the proliferating stage and reinoculated into a mouse it continued to grow actively. After a period of six or eight days' active growth in the mouse it could again be grafted into a rat. This zigzag grafting could be carried on indefinitely with no apparent effect on the tumor tissue or in lessening its activity of growth. The interpretation, suggested by Ehrlich, is that each species provides its tissues with a specific food substance (X) which is necessary for its maintenance and growth. The temporary survival of the mouse tissue in the rat is due to the amount of this specific food carried over with the graft. When this is exhausted the graft dies unless returned to its native species, where it will accumulate a fresh supply of the specific food and again be able to survive for a time in a foreign species.

* Received for publication, March 24, 1914.

[1] Ehrlich, P., *Arb. a. d. k. Inst. f. exper. Therap.,* 1906, No. 1, 84.

The chief opponent of this theory is Bashford[2] who rests his objection on the findings in an experiment in which rats were inoculated a second time with mouse tumor. Under these conditions the second graft, although containing an equal amount of the hypothetical food substance, would survive only two to three days. From this fact he concludes that there is an active immunity developed against the cancer cell as a foreign proteid. The time of survival of the first graft he considers the time required for the development of the active immunity. Bashford[3] claims that the immunity to homoplastic grafting is an entirely different process and that it depends entirely on the blood vessel and stroma reactions. The merits of the two theories will not be discussed; they are quoted to give an idea of the present views on the subject.

LYMPHOCYTIC REACTION IN RELATION TO TISSUE GRAFTS IN IMMUNE ANIMALS.

The occurrence of a lymphocytic reaction around tissue grafts in immune animals has been pointed out by numerous observers[4] and arises whatever the type or source of the animal's immunity. The immune states are: the natural immunity possessed by an animal individually, or because of variety of species; the acquired immunity which is present in animals that have recovered from a primary or implanted tumor; and finally the so called artificial immunity which can be induced by one of several procedures. The small round cell or lymphocytic infiltration is present when there is healing in a spontaneous tumor, and is seen around the edge of slowly growing cancers in man. The importance of these cells in the immunity reaction to tissue grafts would seem evident, yet they have received scant attention.

HETEROPLASTIC GRAFTING IN A NON-RESISTANT ORGANISM, THE CHICK EMBRYO.

In a previous communication[5] it has been pointed out that the avian embryo has no defensive mechanism against the growth of

[2] Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

[3] Bashford, E. F., and Russell, B., *Lancet*, 1910, i, 782.

[4] For the literature see Da Fano, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, v, 1.

[5] Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1912, lix, 874.

tissues of a foreign species. The tumor tissue of a rat, for instance, by transference from embryo to embryo could be kept growing in the chick for an indefinite period.[6] The rat tissue underwent no marked change during its long sojourn in the chick embryo, as was shown by the fact that at any time during this period it could be replanted successfully into its native species but was promptly disintegrated when grafted into the adult chicken. It was later shown[7] that a defensive mechanism developed rapidly in the embryo at about the time of hatching, quickly destroying any foreign tissue that might be present. The foreign species tissue growing in the embryo shows a total absence of a round cell reaction. The lymphoid cells around the graft first become evident at about the time that the defensive mechanism begins to show its effect. Other than this there is no great change in the embryo to account for this sudden development from a susceptible to a highly resistant organism. If this change is the result of the sudden activity of a tissue or organ formerly quiescent, it should be possible to provide the embryo with this necessary tissue or organ by grafting various adult tissues into the chick embryo.[8]

ACTION, IN VITRO, OF TISSUES IN HOMOLOGOUS PLASMA ON THE GROWTH OF HETEROLOGOUS TISSUE.

Lambert and Hanes[9] have shown that the tissues of one species are capable of growth in the plasma of certain other species. As a preliminary step to the experiment suggested above an attempt was first made to determine the interaction of tissues *in vitro*. Bits of a rapidly growing rat sarcoma were placed in drops of chicken plasma and to these were added in series bits of various adult chicken tissues. The cultures were mounted in hollow slides according to the well known method. The rat tissue in chicken plasma grew remarkably well and was not affected by adult chicken connective tissue, kidney, or liver in close proximity. When, however, a bit of adult chicken spleen was growing in the same drop

[6] Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.
[7] Murphy, Jas. B., *idem*, 1914, xix, 181.
[8] Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1914, lxii, 199.
[9] Lambert, R. A., and Hanes, F. M., *Jour. Exper. Med.*, 1911, xiv, 129.

of plasma there was practically a total inhibition of the growth of the rat sarcoma. The only other tissue showing a similar effect was the bone marrow, which caused definite retardation, but not so marked an inhibition as that brought about by the spleen.

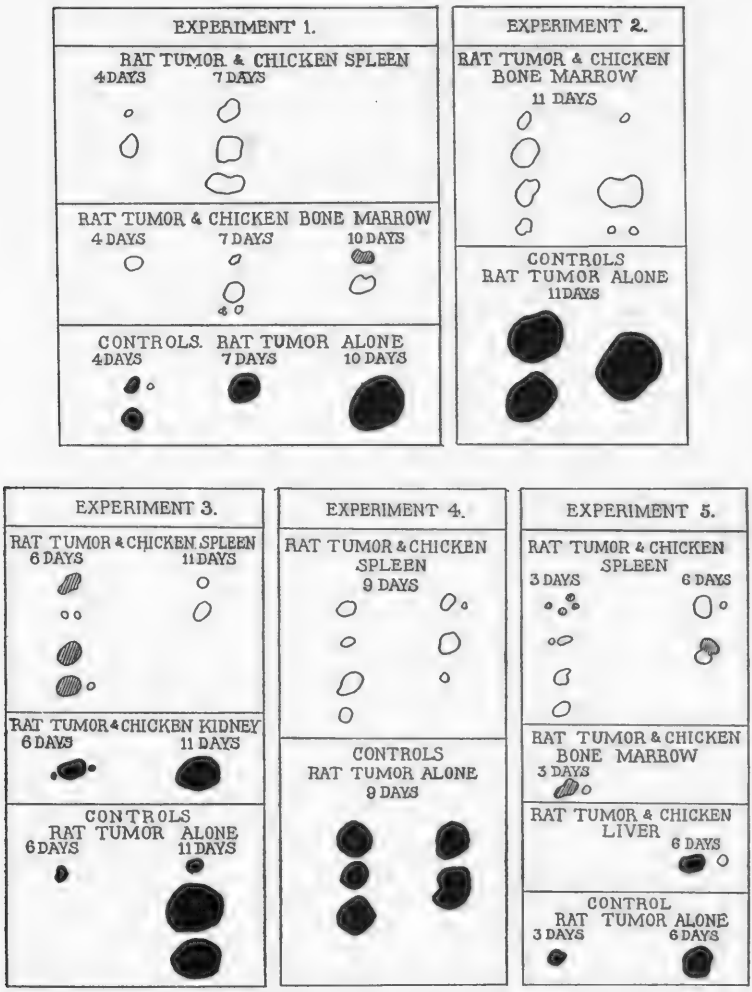
THE EFFECT OF ADULT CHICKEN TISSUE GRAFTS ON THE HETEROPLASTIC GRAFTING IN THE EMBRYO.

Since it is possible to graft various adult tissues into the embryo[10] the above experiment was repeated *in vivo*.

In the first series, comprising twenty experiments and over 150 embryos, grafts of rat sarcoma and bits of adult chicken tissues were placed side by side in the outer membrane of seven day chick embryos, according to the method previously described. The adult chicken tissues used were spleen, kidney, liver, bone marrow, and connective tissue. The eggs were returned to the incubator, and at intervals up to the eighteenth day of incubation part of each lot was opened and the grafts were removed for microscopic examination. Text-figure 1 shows the results in a few of these experiments. In the instances where adult chicken kidney and rat sarcoma were inoculated together the resultant tumors were as large as the controls of rat tumor alone; that is, they generally measured from one to two centimeters. Microscopic examination showed the rat cells in active proliferation, with the kidney tubules, also in good condition, scattered through the tumor mass or in a clump at its edge (figure 1). Chicken liver grafts generally caused a widespread necrosis of the membranes of the chick. When bits of the liver graft survived they were found to consist of a few scattered bile capillaries. If the rat tissue graft escaped the necrosis it was found to be in as active growth as the controls. Connective tissue of the adult chicken had no effect on the rat tumor cells in the embryo, although the connective tissue itself grew well.

The striking result was obtained when grafts of adult chicken spleen were inoculated with the rat tumor. The resulting tumors instead of being well rounded, greyish, and semitranslucent were flat, often mottled, yellowish, and opaque. Microscopic examination of specimens removed after three or four days showed the

[10] Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.



TEXT-FIG. 1. This chart shows in silhouette the results of simultaneous inoculation of rat sarcoma and a graft of adult chicken tissue into the outer membrane of chick embryos. The unshaded nodules were found on microscopic examination to be made up of the adult chicken tissue and reactive tissue, but showed no surviving rat cells. The shaded nodules were found to have a few rat cells embedded in a mass of reactive tissue (figures 2, 3, and 6). The black represents tumors in active growth, with no sign of defensive reaction.

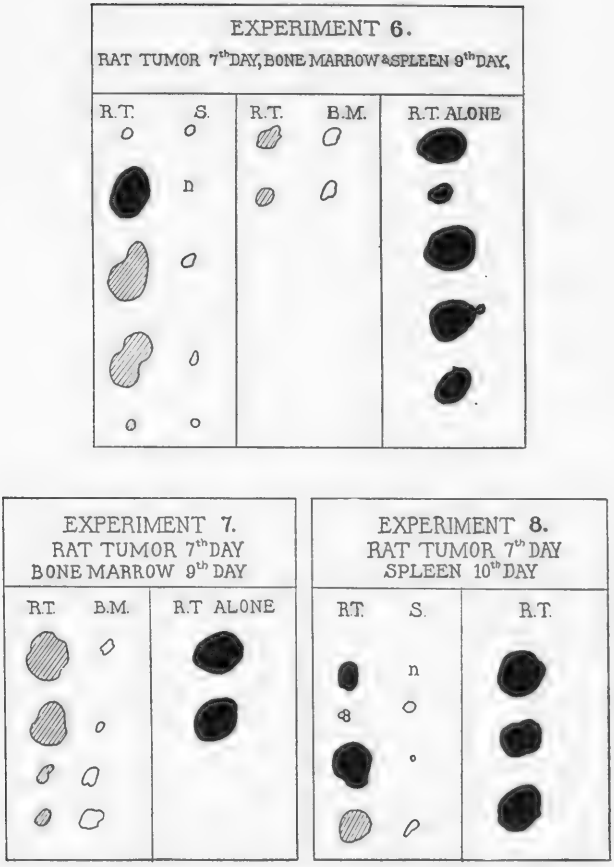
spleen graft well established, made up of the typical spleen cells. The rat tissue was found much degenerated, surrounded by collections of small round cells and largely replaced by connective tissue (figure 2). Later stages show the rat tissue to be dead, embedded in a mass of connective tissue with clumps of small round cells scattered throughout the surrounding tissue (figure 3). This condition offers a strong contrast to the picture shown by the controls of the rat tumor inoculated alone. Here the rat cells are practically devoid of stroma and the edges show no reactive tissue (figure 4). The final stages of rat tumor and chicken spleen showed the spleen graft to be in good condition, but with no evidence of the rat tissue remaining (figure 5).

The action of bone marrow resembled that of the spleen (figure 6), but was slower and less complete. The bone marrow grafts were composed for the most part of fat cells and collections of lymphoid cells.

THE EFFECT OF CHICKEN SPLEEN AND BONE MARROW ON ESTABLISHED GRAFTS OF FOREIGN TISSUES IN THE EMBRYO.

The fact that the tissues in the foregoing experiments were growing side by side and often intermingling presents a difficulty in the interpretation. The present series of experiments was planned to avoid the contact action and to give the spleen and bone marrow a more severe test. The kidney (figure 7) and other tissues mentioned above have no evident effect even in contact and they were therefore not used in this experiment.

Series of eggs were inoculated with rat sarcoma in the usual way on the seventh day of incubation. Two or more days later an opening was made on the opposite side of the eggs, and an adult chicken spleen or bone marrow graft was placed in the outer membrane. Some of the results are shown in outline in text-figure 2. On examination of these specimens eleven days later, the controls inoculated with rat tumor alone showed, almost without exception, large, well rounded, semitranslucent masses at the point of inoculation. In the embryos carrying a graft of adult spleen or bone marrow the tumors were flat, yellowish, and opaque. In some of these only a flake of tumor survived (figure 8). Microscopic ex-



TEXT-FIG. 2. This chart shows in silhouette the effect of adult chicken spleen and bone marrow on established and growing rat tumor in the embryo, when the adult tissues were inoculated at a distance. In the column with double rows of silhouettes the one on the left is the rat tumor (R.T.) and that on the right the bone marrow (B.M.) or spleen (S.) in the same embryo. The last column gives the controls of rat tumor alone. The day of incubation at which the inoculation was made is given in the caption. All tumors were removed at the eighteenth day of incubation. Black indicates that the tumors are composed of rat cells in active proliferation; the shaded outlines, that the rat tissue is much degenerated, with pronounced infiltration with round cells (figures 9 and 10). The unshaded outlines indicate that none of the rat cells survived. N indicates that graft did not take.

amination of the tumors showed massive collections of lymphocytes around the edges and in clumps associated with the blood vessels throughout the tumor (figures 9 and 10). There was a great increase in the connective tissue surrounding and replacing the rat tissue. The rat cells showed many degenerated forms and mitotic figures were rare. It would seem therefore that the adult spleen and bone marrow are capable of supplying a defensive mechanism to the chick embryo, even though the graft of spleen or bone marrow be some distance from the foreign tissue and introduced after the foreign tissue is established and actively growing.

DISCUSSION.

I shall make no attempt to discuss these findings in relation to the theories already brought forward to explain the failure of heteroplastic tumor grafts. The constant result obtained in a score of experiments or more shows conclusively that the adult spleen and bone marrow are capable of supplying a defensive mechanism to the chick embryo, which under ordinary conditions offers no resistance to the growth of foreign species tissue. Furthermore, the embryo bearing such grafts of spleen and bone marrow defends itself in the same way as the adult, if we may judge from the histological picture. The cells common to the graft of bone marrow and spleen, to the reaction around the foreign species graft in the adult, and to the embryo supplied with a defensive mechanism is the small lymphoid cell. It is therefore natural to suppose that this is an active agent in the defense.

Whether or not these lymphoid cells are the important factors in the failure of homoplastic grafts under certain conditions remains to be seen. Certainly a large preponderance of the evidence points in this direction. They are present often in large numbers around grafts of transplantable cancer in immune animals of the same species, regardless of the type of immunity. A recent communication of Apolant's[11] adds weight to this idea. He has shown that in splenectomized animals only a slight or very transient immunity can be developed to transplantable tumors. Oser and Pribram[12]

[11] Apolant, H., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xvii, 219.

[12] Oser, E. G., and Pribram, E. E., *Ztschr. f. exper. Path. u. Therap.*, 1912, ii, 295.

have shown also that transplantable tumors grow more rapidly in splenectomized animals. The results reported by Baeslack[13] indicate that the number of small lymphocytes in the circulating blood of a tumor-bearing animal has a definite relation to the rate of growth of the tumor, falling rapidly in a susceptible animal and increasing steadily in an animal showing resistance.

SUMMARY.

It has been shown that the chick embryo offers suitable conditions for the growth of implanted tissues, whether these be embryonic or adult, of the same species or a foreign one. The chick at about the time of hatching develops a defensive mechanism against the tissue of foreign species. This resistance can be supplied to the embryo in the early stages if grafts of adult spleen or bone marrow are implanted. Under these conditions the embryo exhibits the same resistance to foreign tissue as does the adult, and presents the same histological manifestations about the graft. Furthermore, the same tissues, spleen and bone marrow, when grafted into an embryo with an established and growing rat tumor, bring about a retrogression and absorption of the foreign tissue. Other adult tissues do not supply this power to the embryo.

EXPLANATION OF PLATES.

PLATE 56.

FIG. 1. The edge of a tumor resulting from a ten days' growth of a Jensen rat sarcoma and adult chicken kidney inoculated into the outer membrane of a seven day embryo. The kidney tubules are seen scattered around the edge of the tumor mass, which is made up of the rapidly grown rat cells.

FIG. 2. A section of a tumor resulting from a simultaneous inoculation of adult chicken spleen and a rat sarcoma, after five days' growth in the outer membrane of a chick embryo. A = spleen graft. B = the sarcoma cells surrounded and largely replaced by small round cells and connective tissue.

PLATE 57.

FIG. 3. The remains of the rat sarcoma cells eight days after inoculation into an embryo which at the same time received a graft of adult chicken spleen. The cell structure of the rat tissue is entirely lost and the whole is embedded in a thick connective tissue mass. Small round cells are seen scattered through the section. Compare with figure 4.

[13] Baeslack, F. W., *Ztschr. f. Immunitätsforsch., Orig.*, 1914, xx, 421.

FIG. 4. A section of a tumor resulting from an inoculation of the rat sarcoma alone, after eight days' growth in the chick embryo. Several mitotic figures are seen.

PLATE 58.

FIG. 5. The resulting tumor from a simultaneous inoculation of a seven day embryo with a rat sarcoma and a graft of adult chicken spleen, after eleven days. The spleen graft is seen on the left and the location of the sarcoma graft is on the right. There are no evidences of the rat cells remaining.

FIG. 6. This section shows the effect of a chicken bone marrow graft on a rat sarcoma after six days in the embryo. The rat cells (A) are embedded in a mass of small round cells (B).

PLATE 59.

FIG. 7. A drawing, somewhat enlarged, showing a tumor in the outer membrane of an eighteen day old embryo, resulting from a simultaneous inoculation eleven days previously of grafts of rat sarcoma and adult chicken kidney. The kidney is shown as the bluish nodule in the concavity of the tumor. The controls of rat tumor alone ranged about the same size.

FIG. 8. A drawing, somewhat enlarged, showing the effect of adult chicken spleen on an established graft of rat tumor in a chick embryo. The lower figure is the control of rat tumor alone after eleven days of growth in the outer membrane of chick embryo. The upper figure is the outer membrane of an embryo inoculated at the same time as the above with rat tumor (yellowish area), but two days later a graft of adult chicken spleen was added (pink nodule).

PLATE 60.

FIG. 9. Section of rat tumor in a chick embryo which had at some distance away a graft of adult bone marrow. A = round cell infiltration. B = degenerated rat cells. Compare with figure 4.

FIG. 10. Section of rat tumor in a chick embryo which had at some distance away a graft of adult chicken spleen. A = dense round cell infiltration at edge. B = degenerated rat cells lying in a mass of connective tissue. Compare with figure 4.

FIG. 1.

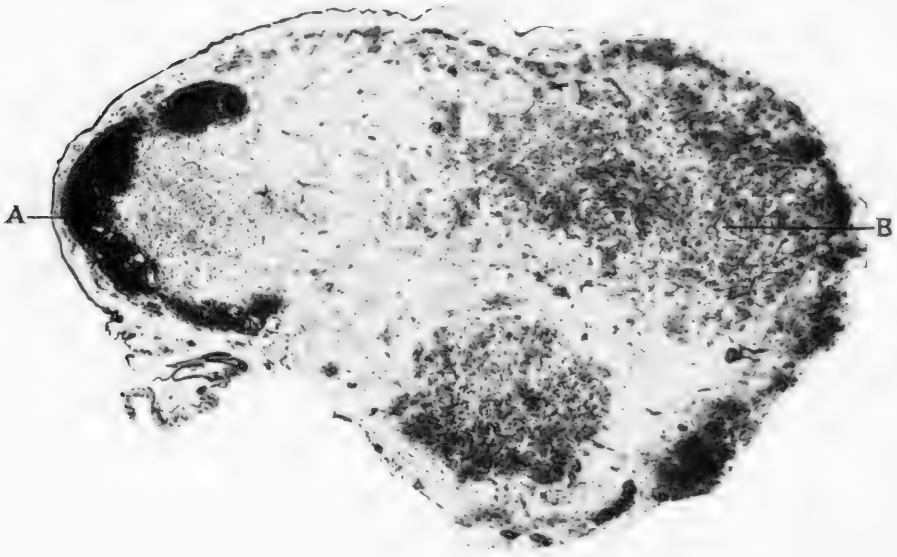


FIG. 2.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

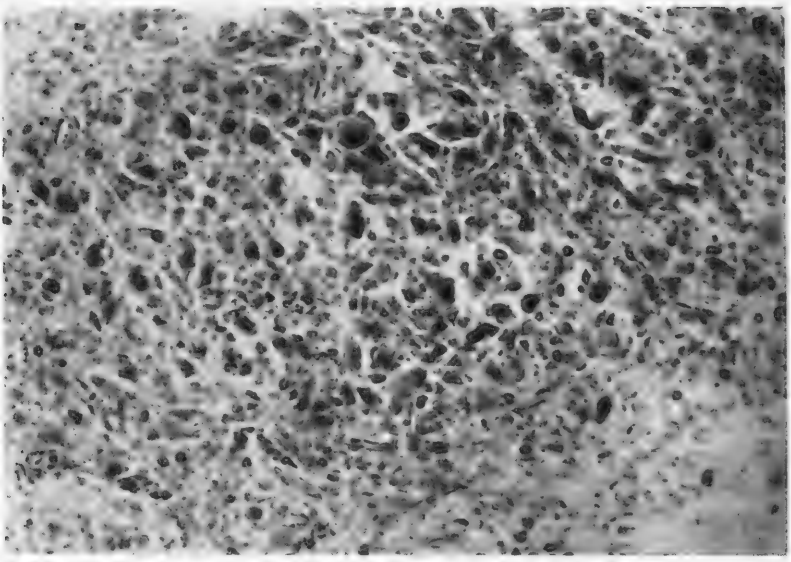


FIG. 3.

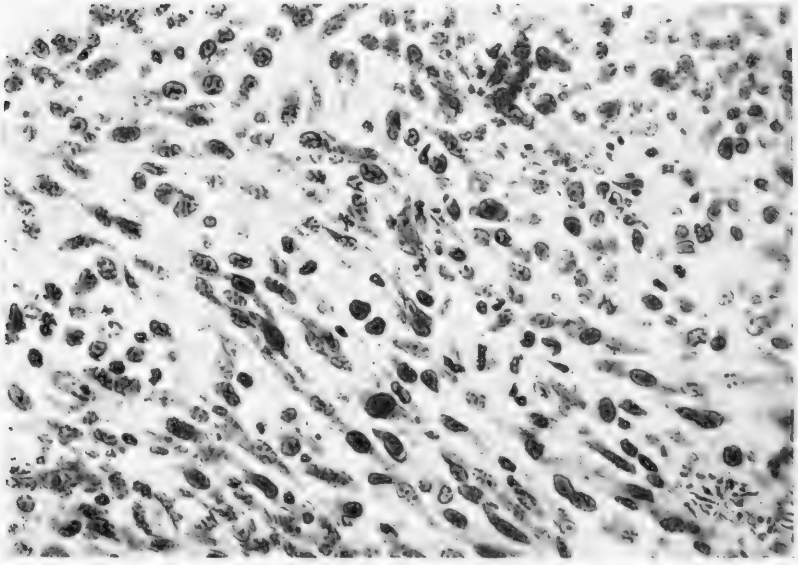


FIG. 4.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

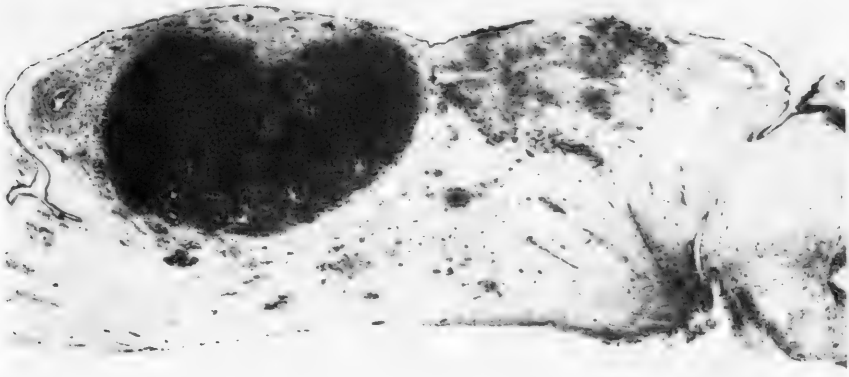


FIG. 5.

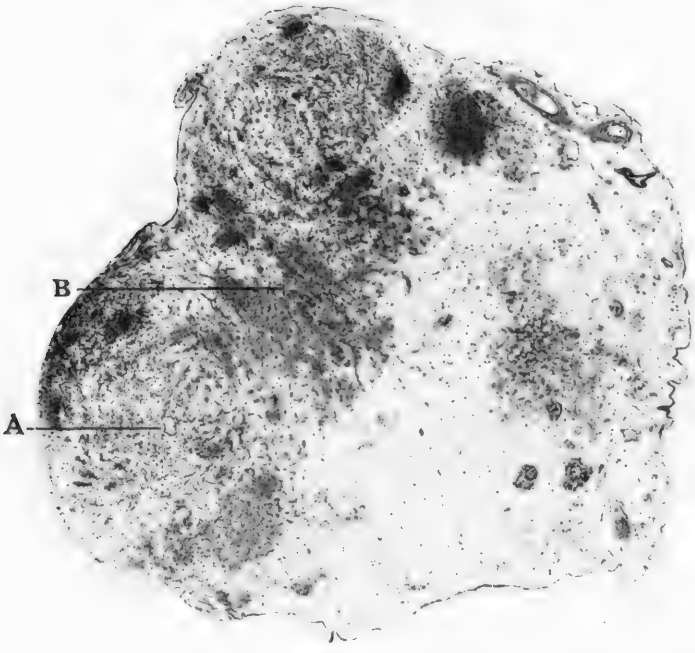


FIG. 6.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

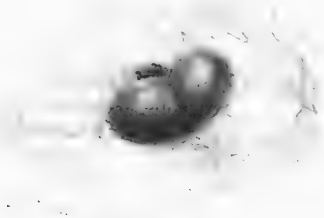


FIG. 7

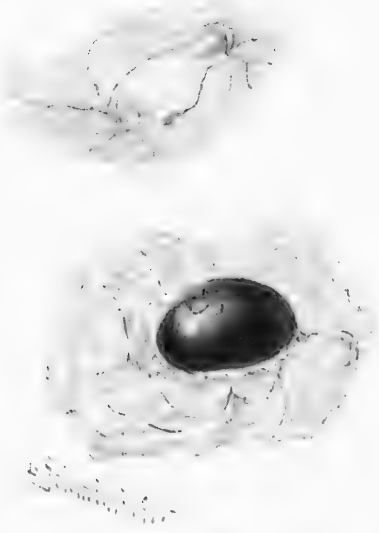


FIG. 8.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

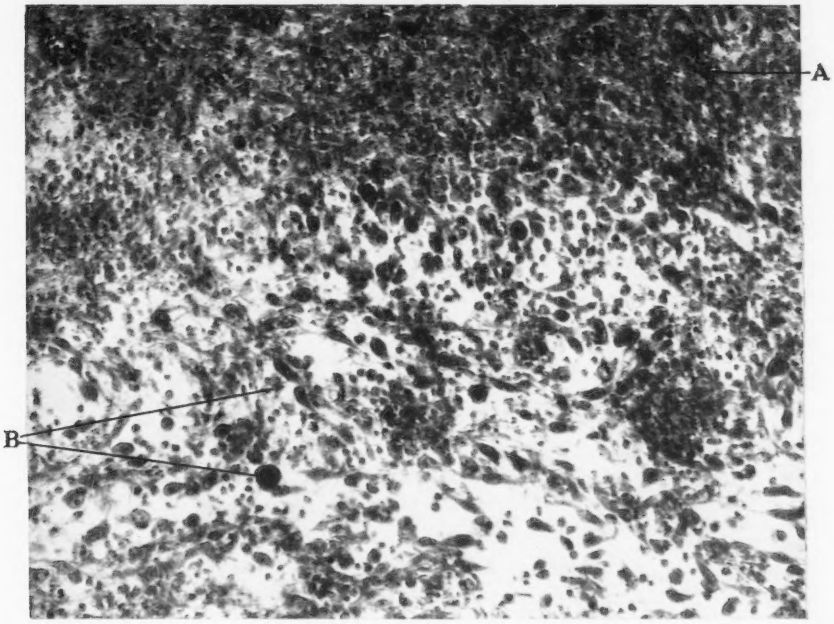


FIG. 9.

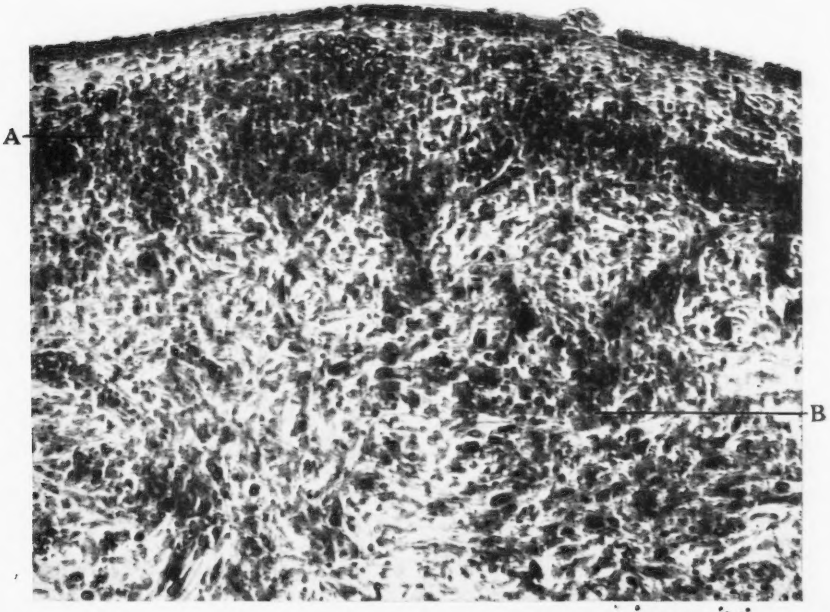


FIG. 10.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)